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ORIGINAL ARTICLE

Apoptosis-Inducing and Antiangiogenic Activity of Partially Purified Protein from the Pericarp of *Zanthoxylum rhetsa in vitro* and *in vivo*

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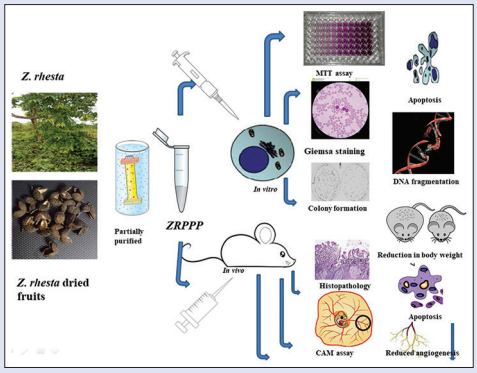
ABSTRACT

Background: Biological activities of *Zanthoxylum rhetsa* have been well studied, and its various parts have been reported to possess anticancer activities. The reports on anticancer activities of proteins from the fruits of *Z. rhetsa* are limited. **Objectives:** The study assessed the proapoptotic and antiangiogenic activity of partially purified proteins from the pericarp of *Z. rhetsa*. **Methods:** MCF-7, MDA MB 231, HeLa, and HCT 116 cells treated with partially purified protein fractions of *Z. rhetsa* pericarp were assessed for cytotoxicity by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-diphenyl tetrazolium bromide (MTT) assay. Fraction ZR3 showed high cytotoxicity against the MCF-7 cells; hence, it was chosen for further assays. The proapoptotic activity was scrutinized by Giemsa staining, acridine orange–ethidium bromide staining, and DNA fragmentation assay. The study was supported by wound healing assay and colony formation assay. Cell cycle analysis was performed by fluorescence-activated cell sorter. Ehrlich ascitic carcinoma-bearing Swiss albino mice were used as *in vivo* model. Angiogenesis was studied by peritoneal angiogenesis in mice and chorioallantoic membrane assay in fertilized eggs. **Results:** MTT assay showed the inhibition of MCF-7 cells (IC_{50} = 21.5 µg/mL) by ZR3 fraction. Reduction in proliferation and failure to produce large cell colonies were observed. Proapoptotic activity was evident from the DNA fragmentation and staining methods. ZR3 blocked the cells in the G2/M phase of the cell cycle. *In vivo* studies suggested the antiproliferative and proapoptotic activities of ZR3. ZR3 exhibited antiangiogenic properties *in vivo*. **Conclusion:** This study confirmed the role of *Z. rhetsa* partially purified proteins as a potential proapoptotic and antiangiogenic agent against the MCF-7 cell line.

Key words: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-diphenyl tetrazolium bromide, Anticancer, chorioallantoic membrane, *in vivo*, MCF-7, vascular endothelial growth factor, *Zanthoxylum*

SUMMARY

- *Zanthoxylum rhesta* partially purified protein could be a potential proapoptotic and antiangiogenic agent and could be easily included in the cancer treatment strategies due to its edible and nontoxic nature



Abbreviations used: *ZRPPP*: *Zanthoxylum rhetsa* partially purified proteins; EAC: Ehrlich ascitic carcinoma; CAM: Chorioallantoic membrane; MVD: Microvessel density; H and E staining: Hematoxylin and eosin staining; NCCS: National Center for Cell Sciences; FACS: Fluorescence-activated cell sorter; VEGF: Vascular endothelial growth factor; NCI: National Cancer Institute.

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BACKGROUND

Cancer cases have been predicted to increase exponentially with an almost 60% hike by 2040.[1,2] The current treatment strategies mostly rely on chemotherapy employing cytotoxic drugs which target cancer cells. Unfortunately, this mode of treatment is also associated with side effects and other secondary complications, including adverse effects on the nervous system, immune suppression, etc.[3,4]

Vinca alkaloids such as vincristine and vinblastine paved the road for the use of phytochemicals for cancer treatment.[5] Ever since plants and their bioactive compounds have been the preferred choice for the development of anticancer drugs, various plant extracts and bioactive compounds are rich in proapoptotic and antiangiogenic compounds. Phytochemicals and secondary metabolites such as flavonoids and phenolic compounds have been communicated to be effective against cancer.[6]

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Zanthoxylum rhetsa, an aromatic spice normally used as a flavoring agent in Asian cooking, is widely dispersed in the tropical and subtropical regions including India, China, and Malaysia.[7] Various parts of this tree have been studied for myriad of medicinal applications; conventionally, *Z. rhetsa* is used as an aromatic, astringent, antimicrobial, antiseptic, and antidiabetic agent. It has been used in the treatment of cholera, dermatosis, toothache, and snake bites. The Kannikar tribe in Tamil Nadu, India, uses the prickly thorns to alleviate breast pain in breastfeeding mothers, the Adi tribe of Arunachal Pradesh, India, uses the plant shoot as a vegetable, while people from the coastal region of Maharashtra, India, use the leaf extract as wormicide.[8]

Compounds obtained from *Z. rhetsa* bark include evodiamine, dihdrovicine, rhestine, and chelerythine, while from fruits include dictamine and arboline.[9] Work involving the study of the cytotoxic effect of quinolone, terpene alkaloids isolated from the root bark of *Z. rhetsa* against stomach cancer cell lines SCL, SCL-6, SCL370 6, SCL-9, Kato-3, and NUGC-4 showed weak cytotoxicity.[10] The cytotoxic effects of the isolated compounds on mouse melanoma cells, B16-F10, were demonstrated in a study involving the extraction of tetrahydrofuran lignans from Z. rhesta bark. Kobusin showed a high percentage of cell death with an IC_{50} value of 112.2 g/mL.[11] Volatile oils from *Z. rhetsa* fruits have been prominent in exhibiting strong cytotoxic activity against lung cancer cell line H460.[12] Alkaloids from conical prickles on the stem bark of *Z. rhetsa* have been investigated for cytotoxicity against colon cancer cell line SW-480, cervical cancer cell line HeLa, and breast cancer cell lines SKBR3 and MDA MB 231.[9] Studies have been undertaken to analyze the anticancer activity of various phytochemicals from the different parts of *Z. rhetsa*.[13] However, fruit pericarps that are usually used in culinary preparations are still not much focused upon. The edible nature of *Z. rhetsa* fruits adds advantage as it can be easily incorporated into the treatment regimen without causing secondary complications.

The current study aims at understanding the proapoptotic and antiangiogenic activity of the partially purified proteins from *Z. rhetsa* fruit pericarp both *in vitro* and *in vivo*.

METHODS

Chemicals and reagents

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-diphenyl tetrazolium bromide (MTT), acridine orange, ethidium bromide, and trypan blue were purchased from SRL, India.

Cell lines and culture medium

Breast cancer cell lines (MCF-7, MDA MB 231), human cervical cancer cell line (HeLa), and human colon cancer cell line (HCT-116) were procured from National Centre for Cell Sciences (NCCS), Pune, India, and cultured in DMEM and McCoy's 5A medium respectively, supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 µg/mL) in humidified condition with 5% CO_2 at 37°C till confluency. Then trypsinized with 0.2% trypsin, 0.02% EDTA, 0.05% glucose in phosphate-buffered saline (PBS). Trypsin and antibiotics were acquired from Gibco and Invitrogen Life Technologies (Paisley, UK), respectively. Swiss albino mice were procured from a private firm, Sri Venkateshwara Enterprises, Bangalore, Karnataka.

Sample collection and protein extraction

Dried fruits of *Z. rhetsa* were collected from the Western Ghats region of India, deseeded, and ground to a fine powder. The plant was authenticated by the Department of Botany, University of Mysore, Mysore, India (voucher specimen number: UOMBOT19ZR9). Pulverized powder of *Z. rhetsa* pericarp (25 g) was subjected to continuous agitation in 200 mL of PBS, pH 7.0 at 4°C for 4 h. This extract was filtered and centrifuged at 10,000 rpm for 10 min at 4°C.[14] The supernatant was used for ammonium sulfate precipitation at different concentrations, 0%–40%, 40%–60%, and 60%–80% and was labeled as ZR1, ZR2, and ZR3, respectively, dialyzed against PBS with dialysis membrane of molecular cutoff 2.5 kDa (Sigma Aldrich), pH 7.0, 4°C. These dialyzed fractions were concentrated by lyophilizing with BioTron vacuum freeze dryer and used to evaluate the cytotoxicity.

Analysis of protein

Lowry's method was used for determining the concentration of proteins with bovine serum albumin (BSA) as the standard reference with trivial modifications. Proteins were separated following the Laemmli method on 12.5% SDS-PAGE. The resultant gel was stained with Coomassie Brilliant Blue R-250. A comparison was made with a standard protein molecular marker.[15,16]

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-diphenyltetrazolium bromide assay

Cytotoxicity of the protein samples (ZR1, ZR2, and ZR3) was analyzed by MTT assay. Cells (1×10^6) were inoculated per well in a 96-well microtiter plate and subjected to different concentrations of the sample (0, 10, 20, 40, 80, 160, and 320 µg) followed by 24-h incubation at 37°C, 5% CO_2 atmosphere. Absorbance was read at 590 nm in a multimode reader, Infinite M200 PRO, TECAN.[17] ZR3 was renamed as *Z. rhetsa* partially purified protein (*ZRPPP*) and this name was used for further assays.

Colony formation assay

MCF-7 cells were cultured 400 cells/well in a 6-well plate and treated with *ZRPPP* (20 µg/mL and 40 µg/mL) for 24 h. Colonies surviving the treatment were fixed with methanol–acetic acid solution and fixed with 0.4% crystal violet. Colonies were observed and photographed at 10× magnification.[18]

Wound healing assay

MCF-7 cells (1×10^6) were allowed to confluent; a scratch of 1 mm size was made using a microtip followed by the removal of media containing cellular debris. Fresh medium along with sample *ZRPPP* (20 µg/mL and 40 µg/mL) was added and incubated for 48 h. Changes in the gap were noted and photographed at 10× magnification using the CatCam 130 microscope camera.[19]

Animal experiments

Swiss albino female mice, 6–8 weeks old weighing around 25 ± 1.5 g, were harbored as per the standard laboratory conditions and fed an animal chow diet with *ad libitum* water throughout the experiment at room temperature with good ventilation and light/dark cycle of 12 h. Animals were acclimatized for 1 week before the initiation of the animal experiments.[20] These experiments were by the regulations set by the Institutional Animal Ethics Committee (IAEC, Approval No: BCP/IAEC/EXTP/04/2018), Bharathi College of Pharmacy, Bharathi Nagara, Mandya district, Karnataka, India.

In vivo studies on tumor development and treatment

Ehrlich ascites carcinoma (EAC) cells are used for the induction of tumor development due to their native mouse origin, highly transplantable

nature, short life span, and 100% malignancy. A predetermined number (1×10^6) of viable cells collected from the donor mice were injected into the peritoneal cavity of the recipient mouse and were left to multiply.[21,22] After 10 days of inoculation, cells were withdrawn from the donor mouse and diluted with saline, and approximately 5×10^6 cells per animal were administered intraperitoneally.[23,24] These animals were utilized for further experiments. Growth was monitored daily by recording the body weights of the mice until the end of the experiment. Cell growth is marked by the increase in weight and the abdominal swelling in mice. Mice were divided into two groups, Group I served as a control group (EAC-bearing, $n = 6$), and Group II received the treatment with *ZRPPP* 25 mg/kg bodyweight of the mouse ($n = 6$). On transplantation on the 7th day, three alternate day doses of the sample were administered intraperitoneally. The survivability of the animals was documented separately.[18,25]

Apoptotic studies

Giemsa staining

MCF-7 cells *(in vitro)* and EAC cells *(in vivo)* were trypsinized from treated and control culture plates and fixed with 3:1 methanol acetic acid solution onto glass slides and stained with 0.1% Giemsa stain. Cells were observed under a light microscope and photographed at 20× magnification using the CatCam 130 microscope camera.[17]

Fluorescence study

Acridine orange–ethidium bromide staining

Acridine orange–ethidium bromide staining was performed as per previously reported protocols, 1×10^5 MCF-7 cells and EAC cells were treated with *ZRPPP* (20 µg and 40 µg) for 24 h, and these cells were stained with acridine orange–ethidium bromide (10 µg/mL) for 2 min with gentle mixing. This suspension was smeared onto a glass slide and examined under a fluorescence microscope and photographed at 40× magnification (Carl Zeiss Axio Imager A2).[26]

DNA fragmentation assay

Genomic DNA was isolated using the phenol–chloroform–isoamyl alcohol method described earlier, MCF-7 cells and EAC cells were centrifuged, and the pellets were processed for DNA isolation. The obtained DNA was electrophoresed on 2% agarose gel and visualized under Gel Doc System (Bio-Rad Universal Hood II) and documented.[19]

Annexin V staining

MCF-7 cells (1×10^5 cells/mL cells) were cultured in 24-well plates and treated with *ZRPPP* (20 µg and 40 µg) and control for 24 h, and the cells were harvested and pelleted. The obtained pellet was resuspended in 50 µL binding buffer containing 0.5 µL Annexin V-FITC followed by dark incubation at 4°C for 30 min. Propidium iodide (PI), 50 µg/mL was added to the binding buffer followed by 5 min incubation, and cells were analyzed by flow cytometry.[27]

Fluorescence-activated cell sorting

The monolayer of the cells was trypsinized and centrifuged to obtain a cellular pellet and washed with PBS; cell pellet was fixed with sheath fluid followed by drop by drop addition of 70% chilled ethanol and further maintaining cells at 4°C for 30 min or overnight. On fixation, cells were centrifuged at 2000 rpm at 4°C for 5 min. The obtained cell pellet was washed and resuspended in sheath fluid containing 0.05 mg/mL PI and 0.05 mg/mL RNase followed by 30 min incubation in dark at room temperature. The cell percentage arrested at various phases of the cell cycle was determined using fluorescence-activated cell sorter caliber (BD Biosciences, San Jose, CA).[17,28]

Angiogenic studies

Vascular endothelial growth factor enzyme-linked immunosorbent assay

Ascitic cells from treated and control mice were diluted to 1:10, and 100 µL of this was used for coating 96-well microtiter plates with a coating buffer overnight at 4°C. These wells were washed and reincubated with anti-vascular endothelial growth factor $VEGF_{165}$ antibodies. Further this reaction mixture was incubated with secondary antibody (goat anti-rabbit IgG) tagged with alkaline phosphatase. This reaction mixture was incubated for 2 h at room temperature; to this, 100 µl of p-nitrophenyl phosphate was added, and the absorbance was measured at 405 nm (Thermo Scientific Varioskan Flash Multimode Reader).[16,23]

Chorioallantoic membrane assay

In ovo chorioallantoic membrane (CAM) assay was executed to determine the antiangiogenic properties of *ZRPPP* on the CAM of fertilized eggs as per the methods described earlier. CAM was photographed.[29] Microvessel density (MVD) was counted in ten fields of vascularized areas under high power, and the mean MVD counted was recorded from the peritoneal linings of control and treated mice. Peritoneal linings obtained were fixed in formaldehyde and blocked into paraffin for sectioning and hematoxylin and eosin (H and E) staining.

Histopathological analysis by hematoxylin and eosin staining

The liver and spleen were aseptically obtained from mice sacrificed by the cervical dislocation.[30,31] Histopathological analysis was done on the liver, spleen, and intraperitoneal tissues. In brief, these tissues were fixed in 4% paraformaldehyde and fixed with paraffin wax; 10 µm sections were made using a microtome. These sections were stained with H and E stain and were observed under a low-power light microscope.[17,19]

Statistical analysis

All the values were indicated as the mean ± standard error of the mean for both the control and experimented studies. Statistical analyses were performed using ANOVA, Student's *t*-test, and Kaplan–Meier analysis using GraphPad Prism 8.0.2 (263) (GraphPad Software Inc, California).

RESULTS

Zanthoxylum rhetsa partially purified proteins show cytotoxic activity

Proteins obtained from *Z. rhetsa* dialyzed fractions – 0%–40%, 40%–60%, and 60%–80% – showed promising cytotoxic activity against the different cell lines of various origins such as human and murine mammary cancer, human colon cancer, and human cervical cancer. 0%–40% (ZR1) fraction showed IC_{50} values of 76.95 µg/mL, 76.99 µg/mL, 97.45 µg/mL, and 88.96 µg/mL for HeLa, HCT-116, MDA MB 231, and MCF-7, respectively; the next fraction 40%–60% (ZR2) showed IC_{50} values of 68.95 µg/mL, 70.31 µg/mL, 79.98 µg/mL, and 68.94 µg/mL for HeLa, HCT-116, MDA MB 231, and MCF-7, respectively. The last fraction 60%–80% (ZR3) IC_{50} values were found to be 23.3 µg/mL, 50.6 µg/mL, 71.16 µg/mL, and 21.5 µg/mL HeLa, HCT-116, MDA MB 231, and MCF-7, respectively [Figure 1a-d]. Among the three dialyzed fractions, 60%–80% fraction showed higher inhibitory percentage against MCF-7 cell line. Thus, ZR3 fraction was chosen for further assays. This fraction showed the presence of four proteins in 12.5% SDS-PAGE. These proteins had molecular weights ~ 45 kDa, ~31 kDa, ~24 kDa, and ~8 kDa [Supplementary Figure 1]. *ZRPPP* will be used to represent the protein fraction ZR3 for results henceforth.

Zanthoxylum rhetsa partially purified proteins inhibits cancer cell proliferation *in vitro* and *in vivo*

Based on the IC_{50} values (IC_{50} >50 µg/mL), MCF-7 cell line was stipulated for further studies. Long-term cytotoxicity was evaluated based on the changes in the reproductive integrity of cells by colony formation assay. At 20 µg concentration, *ZRPPP* showed ~ 66% survival and ~53% at 40 µg concentration as compared to the control [Figure 2a and b]. The interactive property of the cells needed to fabricate contact with surrounding cells and extracellular matrices is displayed by proliferating cells, and this is exploited in wound healing assay. Reduction in the cellular migration to heal the wound created was observed in treated cells as compared to control cells which filled the gap [Figure 2c and d].

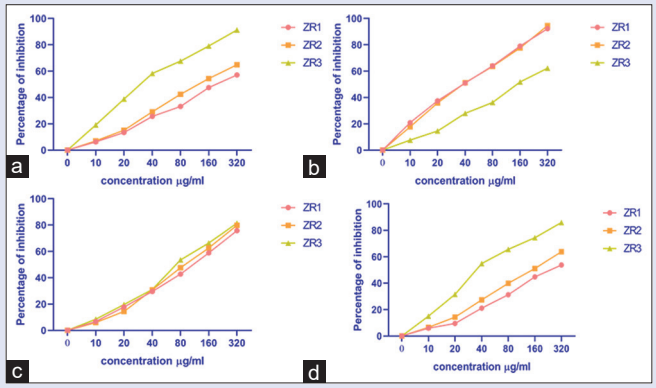

Figure 1: *In vitro* cytotoxic effect of ZR1, ZR2, and ZR3 on (a) HeLa cells, (b) HCT116, (c) MDA MB 231, (d) MCF-7

A dose of 25 mg/kg bodyweight of the animal was administered intraperitoneally to the animals. On completion of the tenure of dose, hematological and serum parameters were analysed. [Supplementary Table 1]. Morphological comparisons between the treated and control mice showed a lack of secondary complications and rendered the compound under study nontoxic for mice treatment. Dynamics in the growth of intraperitoneally inoculated EAC cells were monitored pre- and post-treatment till the animals were sacrificed. It showed a decrease in the tumor weight evident from the decrease in the bodyweight of the *ZRPPP*-treated animals as compared to the control animal [Figure 3a and b].

A comparative decrease in the cell number was observed from the cell count analysis undertaken with a trypan blue dye exclusion study [Figure 3c]. Reduction in the tumor load was also evident from the comparative decrease in the ascitic fluid, 12.7 mL in control, whereas ascetic load decreased to 3.4 mL for the treated mice, thus indicating the cytotoxic effect of ZRPPP [Figure 3d]. Treatment also reflected enhancement in the average survival from 15 days to 30 days [Figure 3e]. Histopathological analysis of liver, spleen, and kidney of treated and EAC-bearing animals showed slight restoration of the normal histological features in the treated organ sections [Figure 4a-f].

Zanthoxylum rhetsa partially purified proteins induces apoptosis and DNA fragmentation *in vitro* and *in vivo*

The formation of apoptotic bodies in the cells treated with ZRPPP was confirmed by Giemsa Staining, indicating that ZRPPP has a proapoptotic effect. This fact was also confirmed by acridine orange-ethidium bromide staining, which clearly showed yellow-green fluorescence in treated cells, indicating apoptosis [Figure 5a and b]. The disintegration

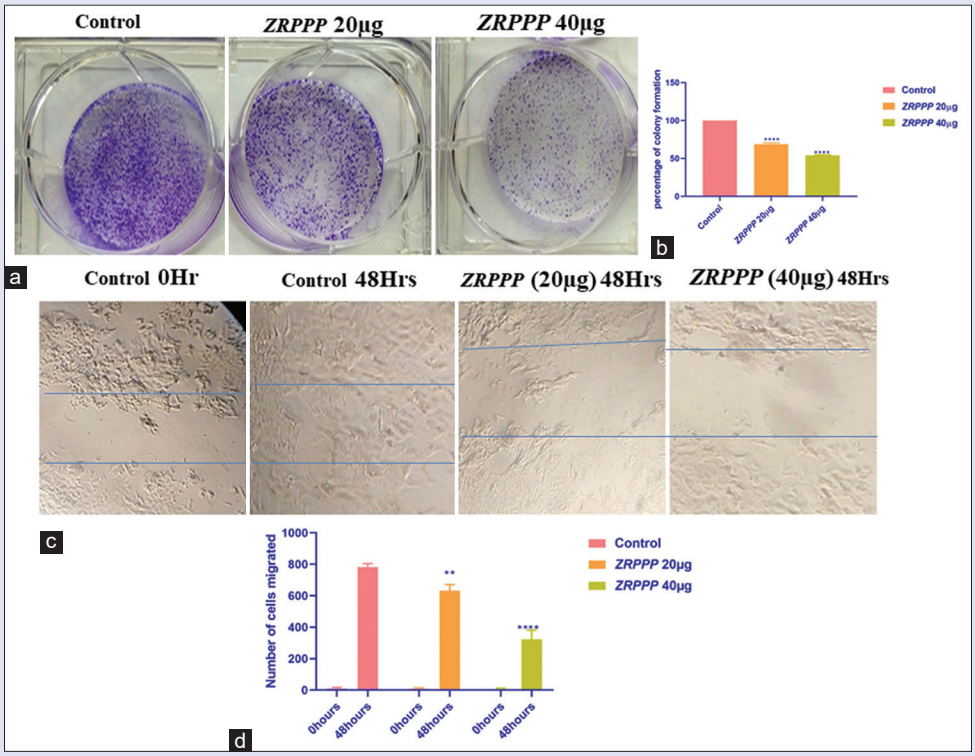

Figure 2: *ZRPPP* inhibits cancer cell proliferation *in vitro* and *in vivo*. (a) Colony formation assay (MCF-7). (b) Graph representing percentage of colony formation. Statistical significance: ***P* < 0.001, *****P* < 0.0001. (c) Wound healing assay (MCF-7 cells), (d) Graph representing migration of cells. Statistical significance: ***P* < 0.001, *****P* < 0.0001. *ZRPPP*: *Zanthoxylum rhetsa* partially purified proteins

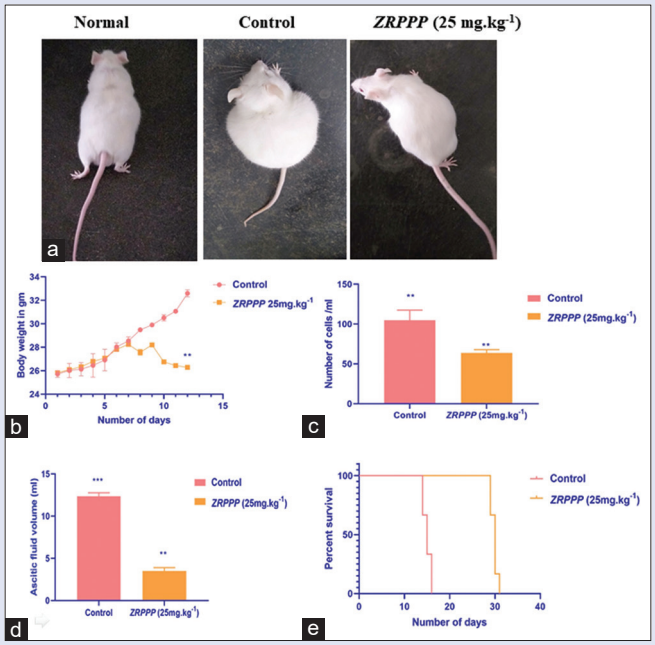

Figure 3: *ZRPPP* reduces EAC cell proliferation *in vivo*. (a) Morphological changes in mice. (b) Graph of bodyweight of treated (25 mg/kg body weight) and control mice. (c) Cell density of viable cells in treated and control mice. (d) Changes in the ascetic volumes of control and treated mice. (e) Kaplan–Meier graph of survivability ($n = 6$) Statistical significance: * $P < 0.05$, ** $P < 0.001$, **** $P < 0.0001$. *ZRPPP*: *Zanthoxylum rhetsa* partially purified proteins; EAC: Ehrlich ascitic carcinoma

of DNA is one of the hallmarks of apoptosis; DNA of MCF-7 cells pretreated with *ZRPPP* showed the fragmentation pattern of DNA as compared to the intact linear DNA in untreated cells, indicating the apoptotic ability of ZRPPP [Figure 5c]. Similar outcomes were evident *in vivo*, EAC cells treated with 25 mg/kg *ZRPPP* per animal; staining of the treated and the control EAC cells showed the typical apoptotic behavior in Giemsa and acridine orange–ethidium bromide staining. Cellular DNA fragmentation was seen in treated mice as compared to the linear DNA in control EAC-bearing mice [Figure 6a and b]. Treated in vivo EAC cells showed apoptotic bodies which are prominent feature of apoptosis. DNA fragmentation was also observed the treated cells [Figure 6c].

Zanthoxylum rhetsa partially purified proteins induced apoptosis and arrested the cells

ZRPPP induced apoptosis, indicated by the percentage of apoptosis in flow cytometry. This was exhibited by the increase in the apoptotic percentage of the treated cells as compared to the control cells. Treated cells showed 0.35% of early apoptotic and 8.05% of late apoptotic cells for 20 µg/mL concentration. The percentage of early apoptotic cells was 0.04 percent and 17.28 percent of late apoptotic cells were found in the treated cells at a concentration of 40 g/mL-1, compared to 0.7 percent of early apoptotic cells and 4.36 percent of late apoptotic cells in the untreated cells [Figure 7c and d]. In conjunction with Annexin-V apoptosis assay, cell cycle distribution analysis showed that ZR impels cell cycle arrest in the G2/M phase at 20 µg/mL, whereas at 40 µg/mL exposure for 24 h, ZR showed an increase in arrested cells in S phase and G2/M phase of MCF-7 cell line [Figure 7a and b].

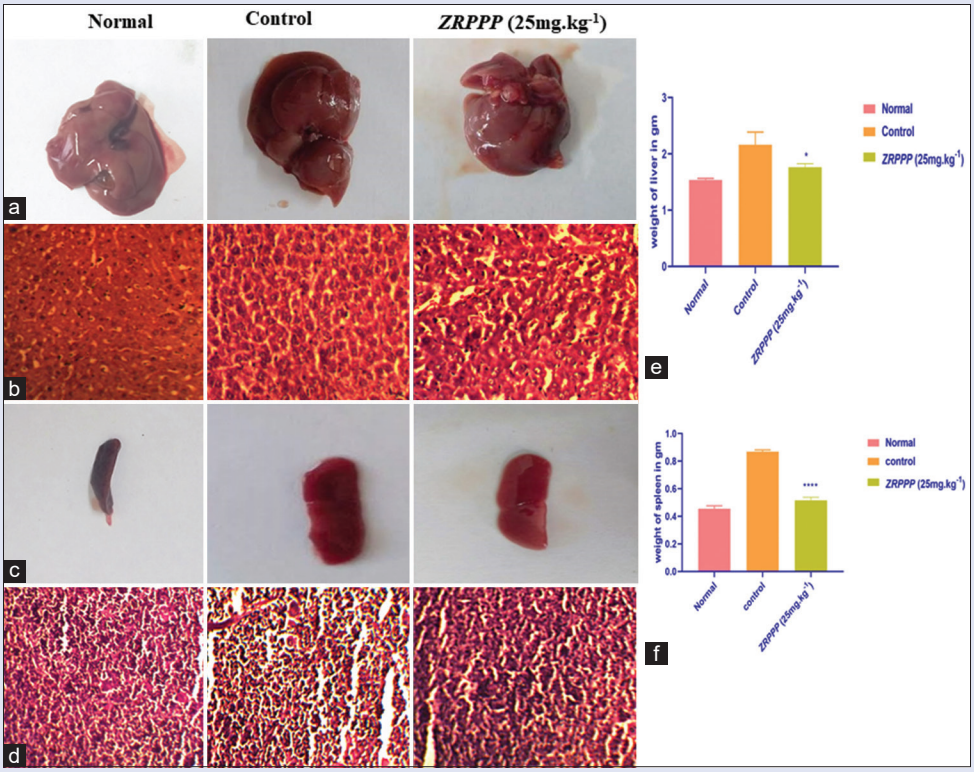

Figure 4: Histomorphological analysis of control and *ZRPPP* (25 mg/kg bw) treatment. (a) Morphological changes in liver. (b) Histopathological analysis of liver. (c) Morphological changes in spleen. (d) Histopathological analysis of spleen. (e) Comparison of changes in weight of control and treated mice liver. (f) Comparison of weights of control and treated mice spleen. Statistical significance: * $P < 0.05$, ** $P < 0.001$, **** $P < 0.0001$. *ZRPPP*: *Zanthoxylum rhetsa* partially purified proteins

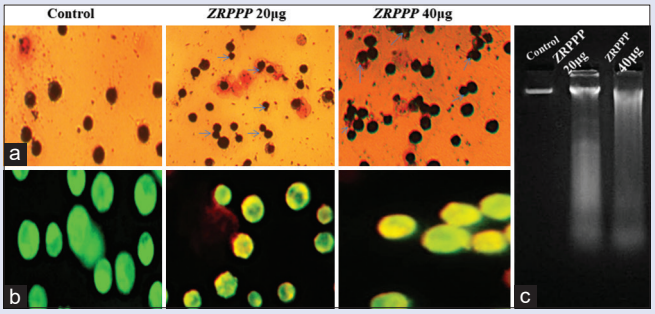

Figure 5: Apoptotic activity of *ZRPPP in vitro* (MCF7 cells). (a) Giemsa staining, (b) Acridine orange–ethidium bromide, (c) DNA fragmentation in treated cells (Lane 1: control, Lane 2: treated). Statistical significance: * $P < 0.05$, ** $P < 0.001$, **** $P < 0.0001$. *ZRPPP*: *Zanthoxylum rhetsa* partially purified proteins

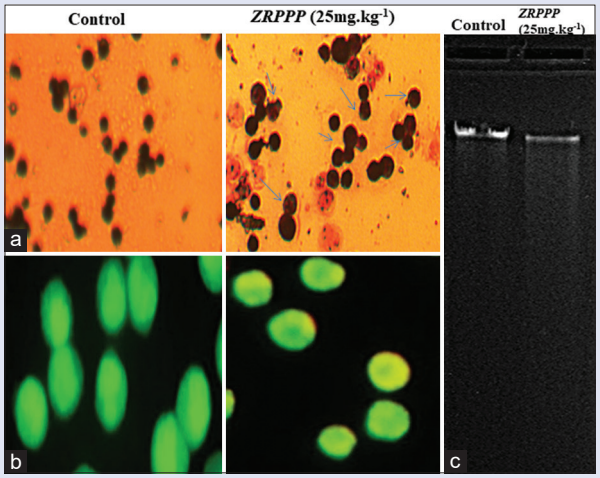

Figure 6: Apoptotic activity of *ZRPPP in vivo*. (a) Giemsa staining, (b) acridine orange-ethidium bromide, (c) DNA (MCF-7 cells) fragmentation assay. *ZRPPP*: *Zanthoxylum rhetsa* partially purified proteins

Zanthoxylum rhetsa partially purified proteins restricts angiogenesis *in vivo* and *in ovo*

Peritoneal angiogenesis in *ZRPPP*-treated and control EAC-bearing mice showed a great decline in the treated animal; further, there was a reduction in the MVD in the treated mice as compared to the control mice. These facts were validated by treating the Swiss albino mice-bearing EAC cells with ZRPPP. The peritoneal tissue from the treated mice was subjected to H and E staining showed a reduction in MVD. Observation of the peritoneal angiogenesis showed a comparative reduction in the angiogenesis in the treated animal as compared to the control animal peritoneum. [Figure 8a-d]. The antiangiogenic property of *ZRPPP* was supported by CAM assay which indicated a reduction in blood vessel formation [Figure 8e and f]. Reduction in the VEGF level as compared to the control indicated the antiangiogenic role of *ZRPPP* [Figure 8g].

DISCUSSION

Spices have been savored since time immemorial as part of cuisines for their flavors and aroma. Moreover, ancient literature reflects their applications as medicines and medicinal preparations. The last few years has seen an immense increase in the prevalence of chronic diseases and also the associated surge in the cost of healthcare commodities. Statistics of 2018 by the World Health Organization reveals cancer as the second leading cause of death globally contributing to 1 in 6 deaths. Moreover, one-third of deaths from cancer are due to dietary risks and lifestyle changes.[32] Complementary and alternative medicine studies include the group of modalities in the treatment regimen which are not part of the standard treatment; thus, the use of the anticancer potential of routine dietary constituents such as fruits, vegetables, herbs, and spices should be considered. Various epidemiological and preclinical studies have inferred the importance of anticancer characteristics of culinary herbs and spices.

Many of the currently available drugs are plant-based drugs, plant peptides, and proteins have turned out to be a critical source of biological compounds that have exhibited bioactivities and can be exploited as a drug.[33] The current study aims at studying the proapoptotic and antiangiogenic potential of partially purified proteins obtained from *Z. rhetsa* pericarp. *Z. rhetsa* is a member of *Rutaceae* family widely dispersed throughout the tropical regions of the world. This family comprises more than 1500 species.[34] This fragrant plant is known to have biological properties such as antioxidant, antimicrobial, and antifungal; further, it has been analyzed for anticancer properties by some workers. Various parts and components from *Z. rhetsa* have already been evaluated for their cytotoxic and anticancer activities. As *Z. rhetsa* seed pericarp is commonly used as a spice in the coastal regions of Western India; a comprised study of its cytotoxicity and related anticancer activity can help in classifying it as complementary and alternative medicine. This study exploited the very fact that pericarp of *Z. rhetsa* is used in culinary preparations and its associated ethnomedicinal properties which could be easily brought into regular usage.

Prevalent anticancer treatments are associated with side effects that ill-treat the normal cells along with the target cells, thus leading to secondary complications associated with synthetic drugs. Thus, the scientific community has been in constant search of anticancer drugs of natural and dietary origin in such a way that could help cancer patients to survive and improve the quality of life. Literature has reflected several studies that have focused on the usage of plant-derived proteins as potential anticancer products that have no or mild secondary complications. This study is aimed at the evaluation of the partially purified *ZRPPP*, which comprises four different proteins ranging from ~ 45 kDa to 8 kDa. *ZRPPP* has shown promising cytotoxic activity against MCF-7 breast cancer cell lines as compared to other cell lines at a lower concentration.

The study also reflected the antiproliferative nature of *ZRPPP* by resulting in a comparative reduction in the formation of large cellular colonies to be formed upon treatment with *ZRPPP*. Treatment also resulted in the failure of migration of cells, thus failing to fill the wound or the gap created. DNA fragmentation resulted in the death of the cells that concluded the proapoptotic activity of *ZRPPP*.

Colony formation by cells is an indication of the reproductive ability of the cell; it determines the ability of each cell to sustain unlimited division. On exposure to the treatment, cells that can express the proteins and DNA undergo one or two cycles of mitosis but cannot produce a large number of progenies and hence considered as reproductive death.[35,36] Yet another and most evident property of cancer cells is invasive proliferation and migration. In wound healing assay, migration of cells toward the "wound" is observed thus filling the gap formed as a result of the wound.

The study was further validated *in vivo* using a Swiss albino mice model inoculated with EAC cells. These cells are one of the most suitable models, because of their mouse origin and easy transplantabilty. The study clearly showed a reduction in the tumor load evident from the changes in the body morphology of the tumor-bearing mice as compared

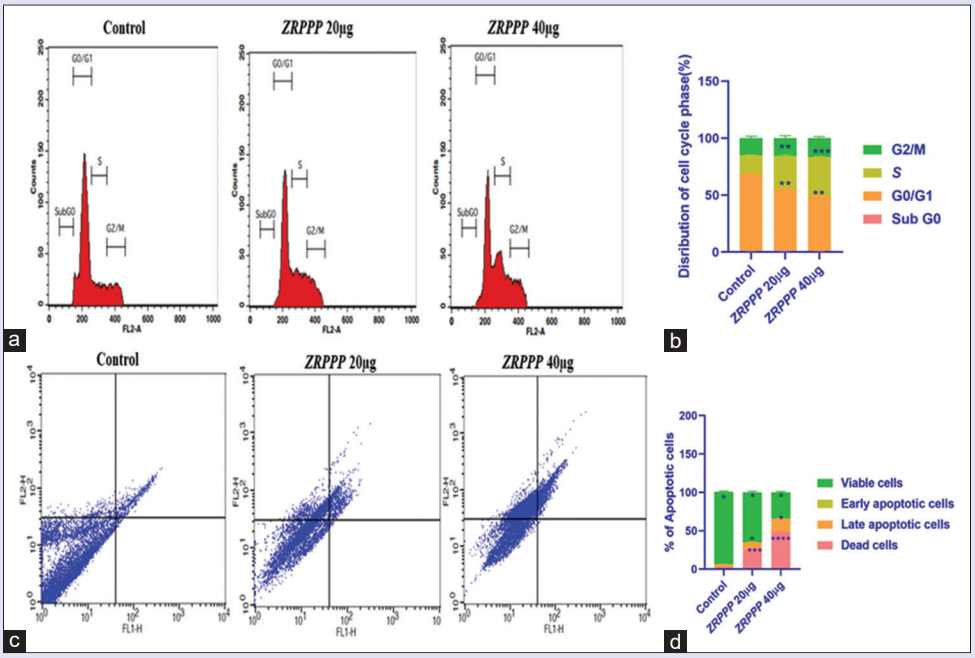

Figure 7: Cell cycle arrest and apoptosis in *ZRPPP*-treated MCF-7 cells. (a) Cell cycle arrest. (b) Graph for distribution of cell cycle phases. (c) Apoptosis in control and *ZRPPP*-treated cells. (d) Graph for percentage of apoptotic cells. Statistical significance: * $P < 0.05$, ** $P < 0.001$, **** $P < 0.0001$. *ZRPPP*: *Zanthoxylum rhetsa* partially purified proteins

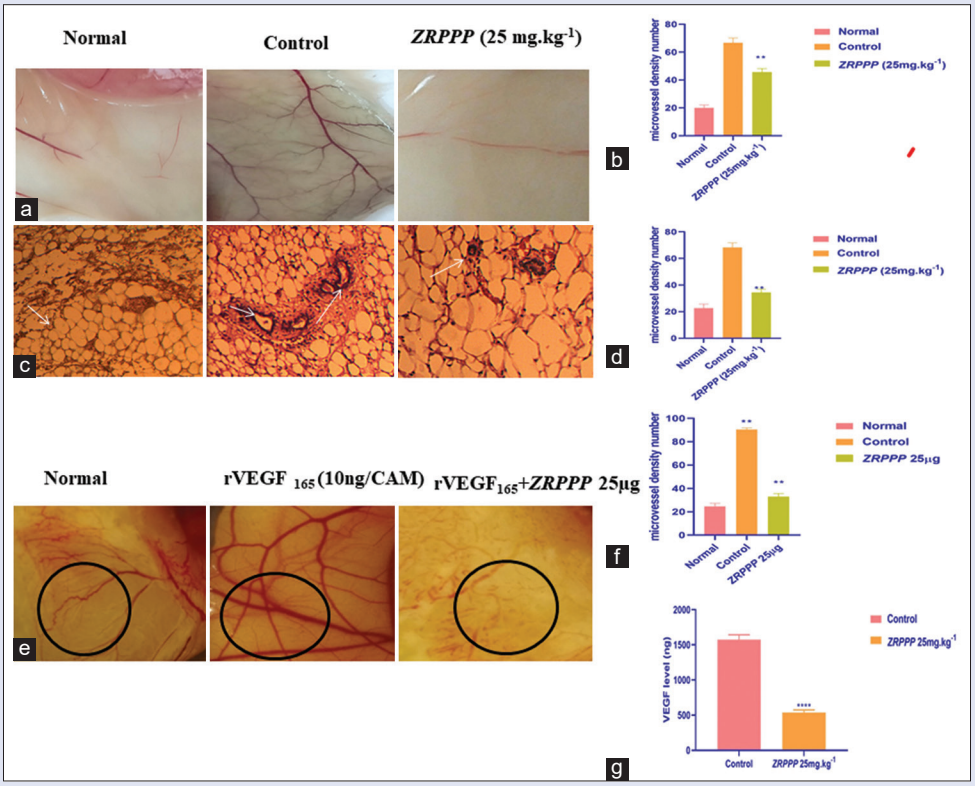

Figure 8: Antiangiogenic activity of *ZRPPP in vivo.* (a) Peritoneal angiogenesis in normal, control, and treated mice. (b) Representative graph for MVD in normal, control, and treated animal peritoneum. (c) Histopathological analysis of MVD of peritoneal tissue. (d) Graphical representation of changes in MVD in the MVD H and E staining. (e) Reduction in angiogenesis in the CAM *in ovo.* (f) Representative graph of MVD in normal, control, and *ZRPPP* 25 µg in CAM. (g) Changes in VEGF levels in control and treated ascites. Statistical significance: * $P < 0.05$, ** $P < 0.001$, **** $P < 0.0001$. *ZRPPP*: *Zanthoxylum rhetsa* partially purified proteins; CAM: Chorioallantoic membrane; MVD: Microvessel density; H and E staining: Hematoxylin and eosin staining; VEGF: Vascular endothelial growth factor

to treated mice bearing the EAC cells. *ZRPPP* was found to be nontoxic as supported by the biochemical parameter studies. *In vivo* study was supported by morphological and histological changes in the organs such as liver and spleen of control and treated animals. This showed negligible changes upon treatment. Further, there was a moderate improvement in the survival of the treated EAC-bearing mice as compared to the EAC-bearing mice.

The study was supported with the proapoptotic activity of *ZRPPP* by staining techniques such as Giemsa and acridine orange–ethidium bromide which exhibited the comparison between the control and the treated cells. A similar study was carried out *in vitro* against the MCF-7 cell line at 20 µg and 40 µg concentrations which indicated the formation of apoptotic bodies and ethidium bromide intercalation with the nuclear material of the apoptotic cells. Acridine orange being membrane-permeable, the apoptotic cells are seen to have a dual-stained appearance.

Moreover, the DNA fragmentation pattern presented by treated EAC and MCF-7 cell lines *in vivo* and *in vitro*, respectively, indicated induction of apoptosis. The proapoptotic activity of *ZRPPP* was also supported by flow cytometric study and cell cycle analysis which established the proapoptotic nature of *ZRPPP*. *ZRPPP* arrested the treated MCF-7 cells in the G2/M phase of the cell cycle, thus showing its efficiency as a potent cell cycle arresting product.

The *in vitro* and *in vivo* pieces of evidence suggested the antiproliferative and proapoptotic activity of *ZRPPP*, thus giving a new outlook to the edible nature of *Z. rhetsa*.

Angiogenesis is another factor that is prominent in cancer; sprouting of new blood vessels is very much essential for tumor growth; hence, it becomes a riveting target for analysis of compounds under study. The hindrance of angiogenesis becomes one of the probable mechanisms which could be employed as an anticancer strategy. Changes in the neovasculature in the peritoneal tissues of EAC-bearing mice indicate the efficacy of the compound. Treatment of EAC-bearing mice with 25 mg/kg body weight with *ZRPPP* resulted in a reduction in the sprouting tendency of blood vessels as compared to the control mice which showed extensive angiogenesis.

VEGF secreted by the EAC cells is the growth factor that promotes angiogenesis; thus, quantification of secreted VEGF becomes one of the important factors that determine the effect of the compound under study on angiogenesis. This study showed a decrease in the secreted VEGF, thus indicating the role of *ZRPPP*. Further, *in ovo* study involving CAM assay indicated a decrease in blood vessel formation. The comparatively reduced MVD also indicated the antiangiogenic nature of *ZRPPP*.

Moreover, there was a restoration of organ morphology in the treated animal and the H and E staining showed negligible usual treatment associated with secondary complications. Although many studies have been attempted to study the role of *Z. rhetsa* as an anticancer agent, limited focus has been given to the edible fruit pericarp of the plant. As the fruit pericarp is routinely used culinary preparation, the major findings of this study further strengthened existing literature supporting anticancer properties of *Z. rhetsa*. In brief, *ZRPPP* can serve as a potent proapoptotic and antiangiogenic product that can be used as an anticancer agent.

CONCLUSION

Z. rhetsa pericarp partially purified proteins showed cytotoxic activity against the human cancer cell lines *in vitro*. The fraction *ZRPPP* showed cytotoxic and antiproliferative activity against the MCF-7 cells *in vitro* and EAC cells *in vivo* in Swiss albino mice. *ZRPPP* was also found to have proapoptotic role and arrested the MCF-7 cells in G2/M. Moreover, *ZRPPP* exhibited antiangiogenic effect *in vivo* and *in ovo*. Thus, this study suggests the potential of partially purified proteins from *Z. rhetsa* fruit pericarp (*ZRPPP*) as a proapoptotic and antiangiogenic agent in cancer treatment.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68:394-424.
2. The Burden of Cancer | The Cancer Atlas. Available from: https://canceratlas.cancer.org/the-burden/the-burden-of-cancer/. [Last accessed on 2020 Sep 08].
3. Frank RC, Parsons GV. Fighting Cancer with Knowledge and Hope. Yale University Press; 2009. Available from: http://www.jstor.org/stable/j.ctt1nq7d3. [Last accessed on 2021 Feb 25].
4. Pucci C, Martinelli C, Ciofani G. Innovative approaches for cancer treatment: Current perspectives and new challenges. Ecancermedicalscience 2019;13:961.
5. Cragg GM, Newman DJ. Plants as a source of anti-cancer agents. Ethnopharmacol 2005;100:72-9.
6. Yong MJ, Tani AZ, Salih FA, Pare R, Norgainathai R, Fong SY. Aqueous leaf extract of Clinacanthus nutans inhibits growth and induces apoptosis via the intrinsic and extrinsic pathways in MDA-MB-231 human breast cancer cells. Pharmacognosy Magazine. 2020;16:689.
7. Hartley TG. New species of *Zanthoxylum* (*Rutaceae*) from New Guinea. Arnold Arbor 1975;56:369-73.
8. Tangjang S, Namsa ND, Aran C, Litin A. An ethnobotanical survey of medicinal plants in the Eastern Himalayan zone of Arunachal Pradesh, India. J Ethnopharmacol 2011;134:18-25.
9. Sreelekha M, Anto NP, Anto RJ, Shafi PM. Cytotoxicity of 6-acetonyldihydrochelerythrin, arnottianamide and 6-(2-hydoxypropyl)-dihydrochelerythrine towards human cancer cell lines. Indian J Chem Sect B Org Med Chem 2014;53:647-51.
10. Ahsan M, Haque MR, Hossain MB, Islam SN, Gray AI, Hasan CM. Cytotoxic dimeric quinolone-terpene alkaloids from the root bark of *Zanthoxylum rhetsa*. Phytochemistry 2014;103:8-12.
11. Santhanam RK, Ahmad S, Abas F, Ismail IS, Rukayadi Y, Akhtar MT, *et al*. Bioactive constituents of *Zanthoxylum rhetsa* bark and its cytotoxic potential against B16-F10 melanoma cancer and normal human dermal fibroblast (HDF) cell lines. Molecules 2016;21:2-3.
12. Theeramunkong S, Utsintong M, Utsintong M, Utsintong M. Comparison between volatile oil from fresh and dried fruits of *Zanthoxylum rhetsa* (Roxb.) DC. and cytotoxicity activity evaluation. Pharmacogn J 2018;10:827-32.
13. Parrikar PN, Srinivas BK, Kattepura Krishnappa D, Jayarama S. *Zanthoxylum rhetsa* crude protein has promising pro-apoptotic and anti-angiogenic properties. Asian J Biol Life Sci 2021;9:334-41.
14. Thomas AS, Saravanakumar R, Gupta *P* V. Evaluation of cytotoxic activity of protein extracts from the leaves of *Morinda pubescens* on human cancer cell lines. Brazilian J Pharmacogn 2017;27:99-104.
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.
16. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.

17. Srinivas BK, Shivamadhu MC, Jayarama S. Angio-suppressive effect of partially purified lectin-like protein from *Musa acuminata* pseudostem by inhibition of VEGF-mediated neovascularization and induces apoptosis both *in vitro* and *in vivo*. Nutr Cancer 2019;71:285-300.
18. Chakkere Shivamadhu M, Srinivas BK, Jayarama S, Angatahalli Chandrashekaraiah S. Anti-cancer and anti-angiogenic effects of partially purified lectin from *Praecitrullus fistulosus* fruit on *in vitro* and *in vivo* model. Biomed Pharmacother 2017;96:1299-309.
19. Srinivasa Balaji K, Shivaprakash P. Angio suppressive effect of *Clitoria ternatea* flower extract is mediated by HIF-1α and down regulation of VEGF in murine carcinoma model. Med Chem (Los Angeles) 2016;6:515-20.
20. Samudrala PK, Augustine BB, Kasala ER, Bodduluru LN, Barua C, Lahkar M. Evaluation of antitumor activity and antioxidant status of *Alternanthera brasiliana* against Ehrlich ascites carcinoma in Swiss albino mice. Pharmacognosy Res 2015;7:66-73.
21. Islam K, Ali SM, Jesmin M, Khanam JA. *In vivo* anticancer activities of benzophenone semicarbazone against Ehrlich ascites carcinoma cells in Swiss albino mice. Cancer Biol Med 2012;9:242-7.
22. Haldar PK, Kar B, Bala A, Bhattacharya S, Mazumder UK. Antitumor activity of *Sansevieria roxburghiana* rhizome against Ehrlich ascites carcinoma in mice. Pharm Biol 2010;48:1337-43.
23. Belakavadi M, Salimath BP. Mechanism of inhibition of ascites tumor growth in mice by curcumin is mediated by NF-kB and caspase activated DNase. Mol Cell Biochem 2005;273:57-67.
24. Thippeswamy G, Salimath BP. *Curcuma aromatica* extract induces apoptosis and inhibits angiogenesis in Ehrlich ascites tumor cells *in vivo*. mySCIENCE 2006;1:79-92.
25. Lingaraju GS, Balaji KS, Jayarama S, Anil SM, Kiran KR, Sadashiva MP. Synthesis of new coumarin tethered isoxazolines as potential anticancer agents. Bioorganic Med Chem Lett 2018;28:3606-12.
26. Kasibhatla S, Amarante-Mendes GP, Finucane D, Brunner T, Bossy-Wetzel E, Green DR. Acridine orange/ethidium bromide (AO/EB) staining to detect apoptosis. CSH Protoc 2006;2006.
27. Pumiputavon K, Chaowasku T, Saenjum C, Osathanunkul M, Wungsintaweekul B, Chawansuntati K, *et al.* Cell cycle arrest and apoptosis induction by methanolic leaves extracts of four *Annonaceae* plants. BMC Complement Altern Med 2017;17:1-11.
28. Badmus JA, Ekpo OE, Hussein AA, Meyer M, Hiss DC. Antiproliferative and apoptosis induction potential of the methanolic leaf extract of *Holarrhena floribunda* (G. Don). Evid Based Complement Altern Med 2015;2015:756482.
29. Gururaj AE, Belakavadi M, Venkatesh DA, Marmé D, Salimath BP. Molecular mechanisms of anti-angiogenic effect of curcumin. Biochem Biophys Res Commun 2002;297:934-42.
30. Lebre F, Bento D, Jesus S, Borges O. Chitosan-based nanoparticles as a hepatitis B antigen delivery system. In: Methods in Enzymology. London: Academic Press Inc.; 2012. p. 127-42. Available from: https://pubmed.ncbi.nlm.nih.gov/22568904/. [Last accessed on 2021 Feb 25].
31. Susin SA, Larochette N, Geuskens M, Kroemer G. Purification of mitochondria for apoptosis assays. Methods Enzymol 2000;322:205-8.
32. Guo Y, Pakneshan P, Gladu J, Slack A, Szyf M, Rabbani SA. Regulation of DNA methylation in human breast cancer. Effect on the urokinase-type plasminogen activator gene production and tumor invasion. J Biol Chem 2002;277:41571-9.
33. Hew C Sen, Khoo BY, Gam LH. The anti-cancer property of proteins extracted from *Gynura procumbens* (Lour.) merr. PLoS One 2013;8:e68524.
34. Pollio A, De Natale A, Appetiti E, Aliotta G, Touwaide A. Continuity and change in the Mediterranean medical tradition: *Ruta* spp. (*Rutaceae*) in Hippocratic medicine and present practices. Ethnopharmacol 2008;116:469-82.
35. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells *in vitro*. Nat Protoc 2006;1:2315-9.
36. Munshi A, Hobbs M, Meyn RE. Clonogenic cell survival assay. Methods Mol Med 2005;110:21-8.