

Zanthoxylum rhesta Crude Protein has Promising Pro-apoptotic and Anti-angiogenic Properties

Priyanka Naik Parrikar¹, Balaji Kyathegowdanadoddi Srinivas¹, Dharmappa Katteppura Krishnappa², Shankar Jayarama^{1,3*}

¹Post Graduate Department of Biotechnology, Teresian College, Teresian Research Center (Affiliated to the University of Mysore), Mysore, Karnataka, INDIA.

²Department of Biochemistry, Chika Aluvara PG Center, Mangalore University, Mangalore, Karnataka, INDIA.

³Department of Studies and Research in Food Technology, Davengere University, Davengere, Karnataka, INDIA.

Submission Date: 15-09-2020; Revision Date: 02-11-2020; Accepted Date: 05-12-2020

ABSTRACT

Zanthoxylum rhesta is a spice tree normally found in the Western Ghats and Asian countries, its fruits are commonly incorporated in regional cuisines as flavoring agents. In this study, the edible nature of this plant has been exploited to establish its pro-apoptotic and anti-angiogenic properties. ZRCP (*Zanthoxylum rhesta* crude protein) was tested against cell lines of different origins, among these cell lines breast cancer cell line, MCF-7 showed the highest cytotoxicity and was carried for further assays. ZRCP was analyzed for both, *in vitro* and *in vivo* effects. This study was supported by assays like MTT assay, apoptotic studies by Giemsa staining, wound healing assay and colony formation assay. The study reflected the pro-apoptotic and anti-angiogenic properties of ZRCP. ZRCP caused apoptosis in MCF-7 cells and also restricted cell growth thus not permitting the cells to proliferate any further. ZRCP also exhibited anti-angiogenic activity *in vivo* and *in vitro*. ZRCP exhibited promising activity *in vitro* and *in vivo* assays performed, this provided promising facts about the utility of crude protein obtained from *Z. rhesta* as a pro-apoptotic and anti-angiogenic agent of natural origin.

Key words: Anti-cancer, Apoptosis, MCF-7, *Zanthoxylum*, Crude, Anti-angiogenic.

Correspondence:

Dr. Shankar Jayarama,
Assistant professor,
Post-Graduation
Department of
Biotechnology, Teresian
Research Foundation
(Affiliated to University
of Mysore), Siddhartha
Nagar, Mysore-570011,
Karnataka, INDIA.
Phone no: +91
9972130598

Email id: shankarbio@gmail.com

INTRODUCTION

Nature has been a great treasure of myriad ethnomedicinal plants that have been used by mankind from the time immemorial. Various medicinal plants and their medicinal importance have been enlisted in various records. Thus, giving hope for the researchers to rely upon nature to search for a panacea for ailments. Among these plants, spices have prime importance for having medicinal properties. The major advantage of focusing on spices and their products as medicinal agents are that they can be included in diet without major secondary complications which are otherwise associated

with synthetic drugs. Workers have already established the anti-cancer properties of various spices and their products, this is of advantage to the Asian countries as they majorly utilize spices in their culinary preparations. *Zanthoxylum rhesta* commonly known as Indian pepper, Indian prickly ash, or Indian ivy-rue, has been already investigated for its multispectral medicinal properties.^[1] Properties of medical importance like antioxidant, anti-bacterial, anti-fungal, anti-diabetic, anti-septic, anti-helminthic and many reports are already been released indicating its anti-cancer properties.^[1-4]

Its role in treating cholera, toothache, dermatosis, snakebites as a part of regional traditional knowledge is already been cited by various workers.^[5] Prior studies have focused on the various parts of *Zanthoxylum rhesta* and their associated phytochemical constituents with different medicinal properties. Amongst these attempts to decipher *Z. rhesta* as a medicinal plant, scientists have focused on its anti-bacterial, anti-cancer, cytotoxic,

SCAN QR CODE TO VIEW ONLINE



www.ajbls.com

DOI: 10.5530/ajbls.2020.9.50

anti-inflammatory properties. Workers have deciphered the phytochemical constituents of *Z. rhesta* bark, prickles, dried fruits, etc.^[6]

Zanthoxylum species in particular are rich in secondary metabolites like lignoids, alkaloids, flavonoids, sterols, terpenes, amides, etc.^[7] Studies have suggested the presence of alkaloids particularly isoquinoline and quinoline in abundance in the trunk and root bark.^[8] Sesamin, xanthyletin and fluroquinolone are also found in abundance, apart from these other secondary metabolites detected are monolignols, coumarins, alkaloids, lignans, etc.^[9]

Petroleum ether extracts and essential oil from *Z. rhesta* fruits have been reported as mosquito repellent and larvicidal products.^[10,11] Studies on volatile oils have indicated the antioxidant, anti-microbial and anti-malarial properties.^[12,13]

Some constituents from the essential oil such as limonene, sabinene, myrcene, α -pinene, γ -terpinene have been reported for their antioxidant, anti-proliferative, anti-mutagenic and also evidences have been cited to enhance the immune system.^[14]

Another study indicated volatile oils from fresh and dried fruits of *Z. rhesta* to possess cytotoxic activity against non-small lung cancer cells.^[15] Quinolone, terpene alkaloids obtained from the root bark of *Z. rhesta* have shown cytotoxic activity against various stomach cancer cell lines like SCL, SCL-6, SCL-9, Kato-3 NUGC-4, etc.^[6] Tetrahydrofuran lignans from the bark of *Z. rhesta* showed cytotoxicity against mouse melanoma origin cells, B16-F10.^[16] Similarly, alkaloids from the conical prickles of the bark of *Z. rhesta* resulted in reduced proliferation of colon cancer cell lines like SW-480, breast cancer cell line SKBR3, MDA MB 231 and cervical cancer cell line HeLa.^[17]

Our study aims to exploit the edibility and regular usage of *Z. rhesta* in culinary preparations as complementary medicine or additive against cancerous proliferation. This attempt was made considering the side effects of the existing synthetic drugs. The product under study being of natural and edible origin the probable side effects could be considered negligible as compared to the synthetic drugs.

MATERIALS AND METHODS

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 obtained from National Centre for Cell Sciences (NCCS), Pune, India and cultured in DMEM medium and McCoy's 5A medium respectively,

with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 μ g/ml) in humidified condition with 5% CO₂ at 37°C till confluency, further trypsinized with 0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS. Trypsin and antibiotics were acquired from Gibco and Invitrogen life technologies (Paisley, UK) respectively. MTT (3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-Diphenyltetrazolium Bromide), acridine orange, ethidium bromide, trypan blue were purchased from SRL, India.

Sample collection and protein extraction

Dried *Z. rhesta* fruits were collected from the Western Ghat region of the Indian subcontinent. The plant was authenticated by the department of botany, University of Mysore, Mysore, Karnataka, India. Fruits were washed and shade dried. These fruits upon deseeding were ground to a fine powder. Ground powder (25gm) was agitated in phosphate-buffered saline at 4°C, pH 7.0 for 4 hr. This extract was filtered and centrifuged at 10,000 rpm at 4°C, for 10 mins.^[18] The obtained crude protein was freeze-dried with BioTron vacuum freeze drier. This crude protein sample was named as ZRCP (*Zanthoxylum rhesta* crude protein)

Analysis of protein

Protein concentration was determined by Lowry's method with bovine serum albumin (BSA) as the standard reference with trivial modifications. Proteins were separated by the Laemmli method on 12.5% SDS-PAGE. The resultant gel was stained with Coomassie Brilliant Blue R-250 (CBB R-250). A comparison was made with a standard molecular protein marker.^[19,20]

MTT assay

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide), was used to study the cytotoxic effect of ZRCP on cancer cell lines. Cells (1x10⁶) were seeded in a 96 well microtiter plate and were subjected to the ZRCP treatment in varying concentrations (0-320 μ g/ml). The assay was conducted as per the standard procedure with minor modifications. This was incubated for 24 hr at 37°C, 5% CO₂ atmosphere. Absorbance was recorded at 590nm in Infinite M200PRO, TECAN multimode reader.

Colony formation assay

MCF-7 cells were seeded into a 6 well plate at the concentration of 400 cells per well and the cells were acclimatized for two hours followed by treatment with ZRCP 20 μ g per well for 24 hr. After 24 hr, fresh media was introduced with 10% fetal bovine serum, cells were kept under culture condition for 10 days. Surviving

colonies were fixed with methanol- acetic acid solution (3:1) and were stained with 0.4% crystal violet, colonies were observed and photographed.^[21]

Wound healing assay

MCF-7 cells (1×10^6 /well) were allowed to confluent in 12 well plates, 1mm scratch was made with a microtip followed by removal of cellular debris. The media was added along with ZRCP 20 $\mu\text{g/ml}$ and the plate was incubated for 48 hr. The migration of cells towards the central 'gap' was observed. Variations in the gap were noted and photographed using CatCam 130 microscope camera.^[22]

Apoptotic studies

Giemsa staining

In vitro (MCF-7) cells and *in vivo* (EAC cells) were trypsinized from treated and control culture plates and mice respectively. They were washed with PBS and fixed with 3:1 methanol-acetic acid solution onto glass slides and stained with 0.1% Giemsa stain. Cells were observed and photographed using CatCam 130 microscope camera.^[23]

Animal Ethical Statement

Swiss albino female mice aged 6-8 weeks with weights $25 \pm 1.5\text{g}$ were maintained as per the standard laboratory conditions. They were fed an animal chow diet with *ad libitum* water throughout the experiment. Good ventilation with light and dark cycle of 12 hr was maintained. These experiments were conducted by the regulations set by the Institutional Animal Ethics Committee (IAEC), (approval No: BCP/IAEC/EXTP/04/2018) Bharathi College of Pharmacy, BharathiNagara, Mandya district, Karnataka, India.

In vivo studies on tumor development and treatment

The tumor was induced in the mouse peritoneal cavity by inoculating the Ehrlich ascites carcinoma cells (EAC). In brief, viable EAC cells from the donor mouse were injected into the peritoneal cavity of the host mouse and allowed to grow, upon 10 days of inoculation, cells were aspirated from this mouse and diluted with saline, approximately 5×10^6 cells/animal were injected intraperitoneally into new mice/ mouse, these animals were used for further analysis.

Growth dynamics of these animals were analyzed by monitoring the changes in the body weight, changes in external morphology like abdominal swelling. These mice were divided into two treatment groups: Group I-control group (EAC bearing) ($n=5$), Group II-received ZRCP 20mg/kg body weight. After seven days of initial transplantation of EAC cells, treatment was initiated for

three alternate days. Morphological changes in the body, body weight changes, survivability was monitored on regular basis and the changes were recorded.^[22,24]

Angiogenic studies

VEGF Enzyme-linked immunosorbent assay (VEGF- ELISA)

Cells were collected from the ascitic fluid of the treated and control mice, diluted to 1:100. This dilution was used for coating the 96 well microtiter plates along with a coating buffer (0.05M sodium bicarbonate, pH 9.6) overnight at 4°C. Upon washing, these wells were incubated with anti-VEGF₁₆₅ anti-bodies followed by a secondary antibody (goat anti-rabbit IgG) conjugated with alkaline phosphatase for 2hr at room temperature. p-nitrophenyl phosphate (p-NPP), 100 μl was added and the reaction was measured at 405nm using a multimode reader.

Chorio-allantoic membrane (CAM) assay

The anti-angiogenic activity of ZRCP was verified by *in vivo* CAM assay by treating the CAM of fertilized eggs with ZRCP as per the methods described earlier. In brief, fertilized eggs were swabbed with 70% alcohol and were maintained in a humidified atmosphere for 10 days. On the 12th day, a window was made in the eggshell under aseptic conditions. rVEGF₁₆₅ (10ng per egg) and/ or ZRCP was airdried on sterile filter paper disc and placed on the exposed CAM. This window was carefully resealed. Changes in angiogenesis were observed after two days of treatment. The changes in the microvessel density on the CAM was photographed.^[25]

Microvessel density (MVD)

Microvessel density (MVD) was quantified in ten different fields of vascularized areas and mean MVD was recorded. These sections were obtained from the peritoneal linings of the control and treated mice. Vascularized areas were monitored and ten fields were counted 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 H&E staining.

Histopathological analysis by hematoxylin and eosin staining

The liver, kidney, spleen and intraperitoneal tissues were subjected to histopathological analysis. Paraffinized tissues were sectioned ($5\mu\text{m}$). These sections were stained with H&E stain and were observed under a light microscope.^[25]

Statistical analysis

Statistical values were expressed as the mean ± SEM for control as well as the experimented subjects. ANOVA, student’s *t*-test, Kaplan Meier analysis were performed using GraphPad Prism 8.0.2(263).

RESULTS

ZRCP has cytotoxic activity

Crude protein obtained from *Z. rhesta* showed cytotoxicity against cancer cell lines of human origin like HeLa, MCF-7, MDA MB 231 and HCT116. ZRCP showed the highest cytotoxicity against the MCF-7 cell line with corresponding IC₅₀ 53.11µg/ml. IC₅₀ for HeLa, HCT116, MDA MB 231 were 83.39 µg/ml, 81.29 µg/ml, 83.38 µg/ml respectively. Thus further assays were carried out on the MCF-7 cell line (Figure 1).

IC₅₀ determination showed the least IC₅₀ value for MCF-7 cell line for ZRPC treated cells as compared to the other cell lines under study, 50 µg concentration dose indicated ~60% survival and at 70 µg ~40% survival as compared to control cells. Cell-to-cell contact is a very important property of cells which helps them to establish contact with neighboring cells and hence signal transduction, this fact is exhibited in wound healing assay. A remarkable decrease was observed in the wound healing capacity of the treated cells whereas the wound or the gap created in the control cell culture well was recruited with the cells. This indicated a reduction in cellular proliferation of treated cells (Figure 2A-B). Also, the reproductive ability of the cell was reduced as evident from the failure of the treated cells to form colonies as compared to the control (Figure 2C-D).

Animal studies conducted in Swiss albino mice showed similar findings as that of *in vitro* studies. Animals subjected to an intraperitoneal dose of 20mg/kg body weight of an animal, upon completion of the experimental period animals were subjected to various studies. These studies showed clearly the anti-proliferative effect of ZRCP. Comparative analysis

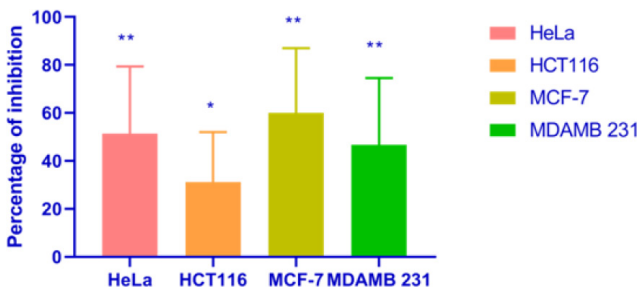


Figure 1: *In vitro* cytotoxic effect of ZRCP A) Cytotoxic effect of ZRCP on A) HeLa cells B) HCT116 C) MDA MB 231 D) MCF-7. Statistical significance: * P < 0.05, **P < 0.001.

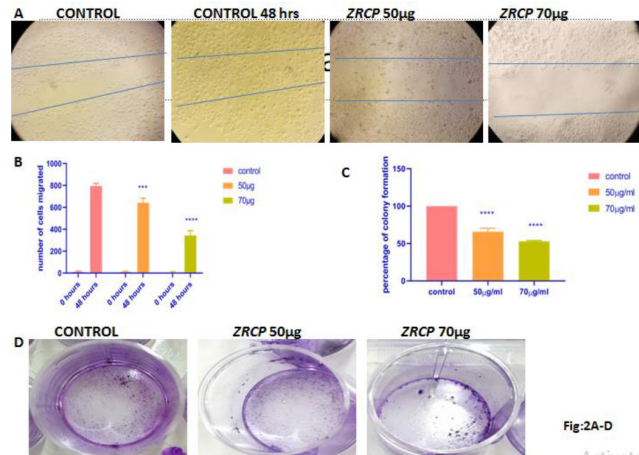


Figure 2: ZRCP inhibits cancer cell proliferation in vitro A) Wound healing assay (MCF-7 cells), B) Graph representing number of cells migrating. Statistical significance: **P < 0.001, **P < 0.0001. C) Graph representing percentage of colony formation, D) Colony formation assays (MCF-7). Statistical significance: **P < 0.001, ****P < 0.0001.**

of hematological and serum parameters showed nil secondary complications which are usually associated with synthetic anti-cancer drugs. Trajectories of changes in the growth pattern of EAC cells were analyzed before and after the treatment till the animals were sacrificed. These experiments clearly showed a decrease in the bodyweight of the ZRCP treated animals in comparison with the control mice (Figure 3A-B).

There was a gradual decrease in the viable cell number obtained from the peritoneal cavity of EAC transplanted mice when analyzed through trypan blue dye exclusion study (Figure 3C) Reduced tumor load was observed to be reduced in the treated animal, this was evident from the diminished ascetic fluid, which was 15.4ml in the control animal whereas 4.6 ml in the treated animal (Figure 3D). Moreover, survival surveys showed an increased life span of the treated animal from 17 days to 26 days as compared to the control animal (Figure E). The toxicological effect of ZRPP on the liver, the spleen was monitored by tracking changes in the organ weight and histopathological analysis by H&E staining of control and treated animals. Slight restoration of the normal histological features in the treated animal organs like liver, spleen, kidney (Figure 4).

ZRCP induces apoptosis in vitro and in vivo

Giemsa staining of the ZRCP treated cells showed apoptotic bodies testifying to the pro-apoptotic action of ZRCP. Apoptotic activity was also supported by *in vivo* studies that showed the presence of apoptotic bodies upon Giemsa staining of ZRCP treated EAC cells as compared to the control cells (Figure 5).

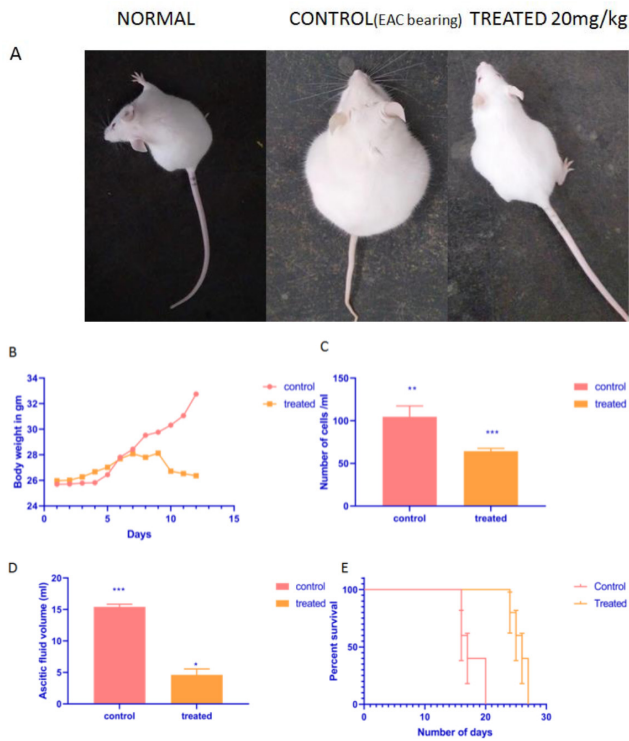


Figure 3: ZRCP reduces cell proliferation of EAC cells *in vivo*. A) Morphological changes in mice B) Representative graph of dynamics in bodyweight of treated (20mg.Kg⁻¹bw) and control mice C) Decrease in the number of viable cells in treated as compared to control mice D) Changes in the ascetic volumes of control and treated mice E) Kaplan –Meier graph of survivability. Statistical significance: * $P < 0.05$, ** $P < 0.001$, **** $P < 0.0001$.

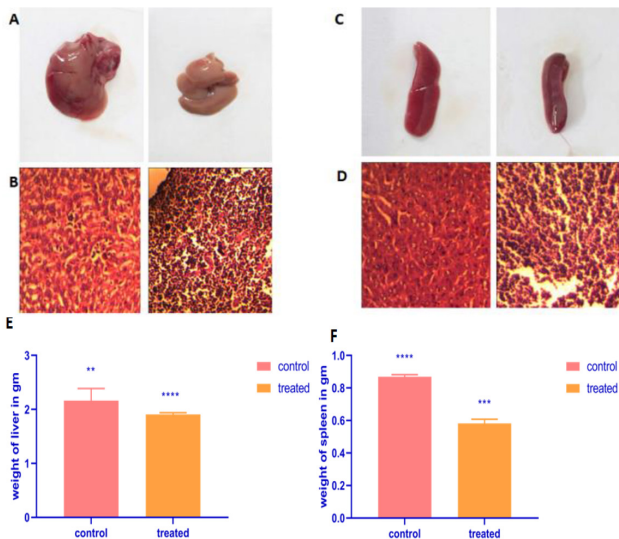


Figure 4: Morphological and histopathological analysis of control and ZRCP (20 mg. Kg⁻¹bw) treatment A) Morphological changes in liver B) Histopathological analysis of liver C) Morphological changes in the spleen. D) Histopathological analysis of spleen. E) Comparative study of changes in weight of control and treated mice liver F) Comparative study of changes in weight of control and treated mice spleen. Statistical significance: * $P < 0.05$, ** $P < 0.001$, **** $P < 0.0001$.

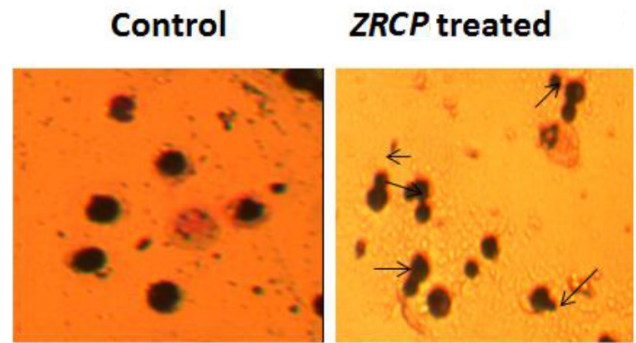


Figure 5: Apoptotic activity of ZRCP *in vitro* (MCF-7 cells) A) Giemsa staining of control and treated cells.

ZRCP restricts angiogenesis *in vivo* and *in vivo*

Angiogenesis in the peritoneal lining of ZRCP treated EAC bearing Swiss albino mice showed a comparable difference between the extent of angiogenesis in control and treated animals. There was reduced neo-sprouting in the treated animal peritoneal whereas the control animal peritoneal showed enhanced angiogenesis. These facts were validated by treating the Swiss albino mice bearing EAC cells with ZRCP and subjecting the peritoneal tissue to section and H and E staining showed a reduction in microvessel density (Figure 6A, 6B, 6C). Further anti-angiogenic role of ZRCP was testified by VEGF quantification, which showed lowered levels of VEGF in treated cells (Figure 6D). Angio-suppressive action of ZRCP was also validated *in vivo* model with CAM assay where a clear reduction in the blood vessel formation was observed in comparison with excessive blood vessel formation in the control egg CAM (Figure 6E).

DISCUSSION

Nature is the biggest source of various raw materials since ancient civilizations men have been dependant on nature for their basic needs and requirements in form of food, shelter and clothing. Slowly men started exploring nature for various purposes in particular as a source of medicines or remedies to cure the ailment. Various categories of plants have been screened for their pro-health activities which have led to commercial exploitation of plants and plant products for mending the health needs of the people. Spices are traditionally used for flavoring food items, especially in Asian countries. Many ancient Ayurveda scriptures have indicated the medicinal properties of many spices that are commonly used thus providing scope for researchers to explore more in this field. Cancer cases have been alarmingly increasing day by day, as per the NIH-

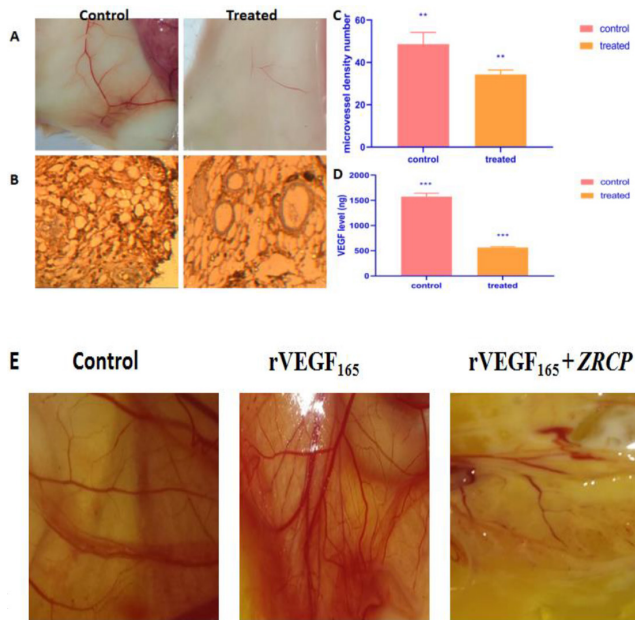


Figure 6: Anti-angiogenic activity of ZRCP in vivo A) Peritoneal angiogenesis in EAC cells bearing Swiss albino control and treated mice. B) Histopathological analysis of microvessel density of peritoneal tissue. C) Comparative graph representing microvessel density in control and treated animals. D) Changes in VEGF levels in control and treated ascites. E) Reduction in angiogenesis in the chorioallantoic membrane *in vivo*. Statistical significance: * $P < 0.05$, ** $P < 0.001$, **** $P < 0.0001$.

National Institute of Cancer 23.6 million cancer cases would arise by 2030.^[26] A study by Siegel and co-workers suggested an average of 1,806,590 new cancer cases and 606,520 cancer deaths in the United States of America in 2020.^[27] Dietary changes and lifestyle changes in recent years have also contributed to over one-third of cancer deaths.^[28,29] Fruits, vegetables, herbs, spices have been suggested to be included in the routine diet by complementary and alternative medicine studies. The present treatment majorly focuses on the use of synthetic drugs but it has been noticed that the associated side effects and secondary complications are numerous, thus there is an alarming need for some alternative which could lower these complications. Plant-based products are increasingly used for the treatment of cancer, among these peptides and proteins have been focused as the next promising drugs or drug sources.^[30] Vinca alkaloids, vinblastine, vincristine are amongst the first plant-based products to be successfully used as anti-cancer drugs, thus these success stories have prompted many workers in the science community to work in the ethnomedicinal and plant-based anti-cancer research.^[31] The hallmarks described by Hanahan mainly focus on apoptosis and angiogenesis as some of the important factors actively affected in cancer.^[32] Thus targeting

such hallmarks effectively can help in understanding the causal root and treatment strategy for cancer. Earlier literature and studies on *Zanthoxylum rhesta* and its traditional applications in medicine prompts to focus on its anti-cancer activity. Most importantly the edible nature of *Z. rhesta* can be exploited as it can be easily included in the diet without any secondary complications. *Zanthoxylum rhesta*, the plant under the present study is a member of the *Rutaceae* family which is majorly found in the tropical regions, in India it is prominently found in the Western ghat region hence it has become a part of culinary preparations in these regions. More than 1500 species have been recorded under this family.^[33] This plant is particularly famous for its aroma, its various parts have been reported for anti-microbial, anti-fungal, antioxidant, cytotoxic, anti-cancer activities. Moreover, traditionally it has been used for various medicinal purposes by many Indian tribes. Secondary complications and associated side effects majorly go hand in hand with the synthetic anti-cancer drug regime. Thus drugs of natural or dietary origin would be preferred candidates. In this study ZRCP, the crude protein obtained from *Z. rhesta* fruit pericarp was analyzed for cytotoxic activity and hence for pro-apoptotic and anti-angiogenic nature against various cancer cell lines. MCF-7 cell line was targeted as it showed the lowest IC_{50} values as compared to other cell lines against ZRCP.

A cell's reproductivity reflects its ability to form a colony and also its ability to undergo unlimited division, even though the cells are exposed to the treatment, some of the cells which can still express the proteins and DNA may undergo one or two cycles of cell division but the colony formation is restricted to a small number of progenies and hence are rendered reproductively dead.^[34,35] ZRCP treatment showed a reduction in the number of colonies formed when compared with the colonies formed by control cells. Cancer cells are known to have invasive and migratory nature which helps them to move to secondary locations in the body, this proliferative and migratory property of cells was analyzed by wound healing assay, control cells filled the wound created in the monolayer whereas the treated cells failed to fill this gap indicating that the treatment has restricted the proliferation of the cells.

In vivo studies also supported the anti-proliferative nature of the EAC cells, Swiss albino model was used as it has a higher rate of translatability in immuno-competent mice. Cells were allowed to grow in the intraperitoneal region of the mice and a specific number of EAC cells were transplanted in the host mouse and further assays were carried out. The study showed a reduction in tumor

load and noticeable changes in the body morphology in comparison with that of the control mice. Biochemical parameters showed non-toxicity of *ZRCP* thus nullifying the doubts of toxicity. Also, histological and morphological studies showed negligible changes in the organs of the treated animals thus strongly supporting the non-toxicity of *ZRCP*. The study also suggested improvement in the survival rate of treated mice in comparison with the control EAC bearing mice.

The pro-apoptotic nature of *ZRCP* was also validated by Giemsa and ethidium bromide staining. MCF-7 cells treated *in vitro* with *ZRCP* showed typical apoptotic bodies formation representing the pro-apoptotic nature of *ZRCP* also in Giemsa stain apoptotic cells showed dual staining.

The anti-angiogenic nature of *ZRCP* was very much evident from the reduced sprouting of blood vessels in treated mice peritoneum. Peritoneal angiogenesis was monitored in treated and control mice which clearly showed the changes, also the quantification of VEGF in the peritoneal fluid indicated reduced VEGF concentration hence marking the efficiency of *ZRCP* in controlling angiogenesis hence providing hope that it could be used as an anti-angiogenic compound. The anti-angiogenic ability of *ZRCP* was also scrutinized *in vivo* by performing CAM assay in which egg with treated CAM showed a reduction in the extent of neo-angiogenesis in comparison with that of the control egg. Thus providing a cumulative result that *ZRCP* has both pro-apoptotic and anti-angiogenic activity and due to its dietary and/or edible origin could be easily incorporated in the human diet.

CONCLUSION

Study on *Z. rhesta* crude protein emphasizes on the cytotoxic, pro-apoptotic as well as anti-angiogenic properties. *Zanthoxylum* genus is worked upon by many workers, work primarily focused on the inedible parts of the plant like prickles, bark, etc. But pro-apoptotic and anti-angiogenic studies of the fruit pericarp concerning proteins are the first attempt made by this study. This study screened different human cancer cell lines for the action of *ZRCP*, amongst the cell lines, *viz.*, HCT116, HeLa, MDA MB 231 and MCF-7 the highest cytotoxicity was observed for the MCF-7 cell line *in vitro*. Hence further evaluatory assays were performed on the MCF-7 cell line. *ZRCP* treatment hindered the proliferative nature of the MCF-7 cell line evident from the wound healing assay where the cells failed to cover the 'wound' made in comparison with the filled or healed 'wound'. Also, the cells failed

to form large colonies when compared with control cells which formed large colonies. Apoptosis was very much evident in Giemsa staining which clearly showed the presence of apoptotic bodies thus emphasizing more on pro-apoptotic nature. Apart from *in vitro* validation, *ZRCP* was subjected to *in vivo* assays also where Swiss albino mice were used as model animals, EAC cells were transplanted in these animals. Growth dynamics of EAC cells were directly reflected by the changes in body weight and external body morphology, these findings were further strengthened by Giemsa staining of the EAC cells which showed the presence of apoptotic bodies in the treated cells. Also, *ZRCP* was rendered non-toxic as suggested by normal biochemical parameters and also the histopathological studies, which showed no secondary complications. A moderate increase in survival was observed in the treated animals as compared to the control ones. The study also shed light on the anti-angiogenic role of *ZRCP*, peritoneal angiogenesis in the treated and control mice showed the comparison between the extents of angiogenesis, further VEGF quantification showed reduced levels in the ascitic fluid of the treated mice. The angiogenic study was supported by *in vivo* analysis in CAM assay, this assay also showed reduced angiogenic proliferation in treated egg CAM. All these evidences firmly support the pro-apoptotic and anti-angiogenic activity of *ZRCP* and hence providing more scope for further analyzing *Z. rhesta* fruit pericarp for pro-apoptotic and anti-angiogenic properties.

ACKNOWLEDGEMENT

The authors acknowledge the Teresian Research Foundation and the P.G. Department of Biotechnology, Teresian College (Affiliated to the University of Mysore) for providing animal cell culture facility. The authors would like to thank the Department of Pharmacy, Bharathi College of Pharmacy, KM Doddi, Mandya, Karnataka for providing the animal house facility.

CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

REFERENCES

1. *Zanthoxylum rhesta* (Roxb.) DC. 2020. Available from: <https://indiabiodiversity.org/species/show/20189>
2. Tangjitjaroenkun J, Supabphol RCW. Antioxidant effect of *Zanthoxylum limonella* Alston. *J Med Plants Res.* 2012;6(8):1407-14.
3. Esterhuizen LL, Meyer RDI. Antimicrobial compounds from *Coleonema album* (Rutaceae). *Z Naturforsch C.* 2006;61(7-8):489-98.
4. *Zanthoxylum rhesta*: Useful Tropical Plants. 2020. [Internet]. [cited 2020 Oct 14]. Available from: <http://tropical.theferns.info/viewtropical.php?id=Zanthoxylum+rhesta>
5. Singh A, Singh RK, Bharadwaj R, Singh AK. Adaptations of culturally and nutritionally important foods in Eastern Himalaya: A case study with Adi women of Arunachal Pradesh. *Indian J Tradit Knowl.* 2014;11:623-33.
6. Ahsan M, Haque MR, Hossain MB, Islam SN, Gray AI, Hasan CM. Cytotoxic dimeric quinolone-terpene alkaloids from the root bark of *Zanthoxylum rhesta*. *Phytochemistry.* 2014;103:8-12. Available from: <http://dx.doi.org/10.1016/j.phytochem.2014.03.008>
7. Javier PLO, Angelica PRJ, Enrique CSL. *Zanthoxylum* Genus as Potential Source of Bioactive Compounds. *Bioact Compd Phytomedicine.* 2012.
8. Tantapakul C, Phakhodee W, Ritthiwigrom T, Yossathera K, Deachathai S, Laphookhieo S. Antibacterial compounds from *Zanthoxylum rhesta*. *Arch Pharm Res.* 2012;35(7):1139-42.
9. Ahsan M, Zaman TA, Hasan CM, Ito C, Islam SKN. Constituents and cytotoxicity of *Zanthoxylum rhesta* stem bark. *Fitoterapia.* 2000;71(6):697-700.
10. Das NG, Baruah I, Talukdar PKDS. Evaluation of botanicals as repellents against mosquitoes. *J Vect Borne Dis.* 2003;40(1/2):49-53.
11. Rabha B, Gopalakrishnan R, Baruah ISL. Larvicidal activity of some essential oil hydrolates against dengue and filariasis vectors. *Eur J Med Res.* 2012;1(1):14-6.
12. Charoenying P, Laosinwattana C, Phuwiwat WLJ. Biological activities of *Zanthoxylum limonella* Alston fruit extracts. *KMITL Sci J.* 2008;8(1):12-5.
13. Wannissorn B, Jarikasem S, Siriwangchai T, Thubthimthed STS. Antibacterial properties of essential oils from Thai medicinal plants. *Fitoterapia.* 2005;76(2):233-6.
14. Bhalla Y, Gupta VKJV. Anticancer activity of essential oils: A review. *J Sci Food Agric.* 2013;93(15):3643-53.
15. Theeramunkong S, Utsintong M, Utsintong M, et al. Comparison between Volatile Oil from Fresh and Dried Fruits of *Zanthoxylum rhesta* (Roxb.) DC. and Cytotoxicity Activity Evaluation. *Pharmacogn J.* 2018;10(5).
16. Santhanam RK, Ahmad S, Abas F, Ismail IS, Rukayadi Y, Akhtar MT, et al. Bioactive constituents of *Zanthoxylum rhesta* bark and its cytotoxic potential against B16-F10 melanoma cancer and normal human dermal fibroblast (HDF) cell lines. *Molecules.* 2016;21(6):2-3.
17. Sreelekha M, Anto NP, Anto RJ, Shafi PM. Cytotoxicity of 6-acetyl-dihydrocherythrin, amottianamide and 6-(2-hydroxypropyl)-dihydrocherythrine towards human cancer cell lines. *Indian J Chem Sect B Org Med Chem.* 2014;53(5):647-51.
18. Thomas AS, Saravanakumar R, Gupta PV. Evaluation of cytotoxic activity of protein extracts from the leaves of *Morinda pubescens* on human cancer cell lines. *Brazilian J Pharmacogn.* 2017;27(1):99-104. Available from: <http://dx.doi.org/10.1016/j.bjp.2016.08.003>
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951;193(1):265-75.
20. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227(5259):680-5.
21. Srinivas BK, Shivamadhuc 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(2):285-300. Available from: <https://doi.org/10.1080/01635581.2018.1540714>
22. 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(23-24):3606-12. Available from: <https://doi.org/10.1016/j.bmcl.2018.10.046>
23. Srinivasa BK, Shivaprakash P. Angio Suppressive Effect of *Clitoria ternatea* Flower Extract is Mediated by HIF-1 α and Down Regulation of VEGF in Murine Carcinoma Model. *Med Chem.* 2016;6(7):515-20.
24. Srinivas BK, Shivamadhuc MC, Jayarama S. *Musa acuminata* lectin exerts anti-cancer effects on HeLa and EAC cells via activation of caspase and inhibitions of Akt, Erk, and Jnk pathway expression and suppresses the neoangiogenesis in in-vivo models. *Int J Biol Macromol.* 2020; S0141-8130(20)34925-4. doi:10.1016/j.ijbiomac.2020.10.272
25. Chakkere SM, Srinivas BK, Jayarama S, Angatahalli CS. Anti-cancer and anti-angiogenic effects of partially purified lectin from *Pracitrus fistulosus* fruit on *in vitro* and *in vivo* model. *Biomed Pharmacother.* 2017;96(10):1299-309.
26. Cancer Statistics. Available from: <https://www.cancer.gov/about-cancer/understanding/statistics>
27. Siegel RL, Miller KDJ. Cancer statistics. *CA Cancer J Clin.* 2020;70(1):7-30.
28. Brennan SF, Cantwell MM, Cardwell CR, Velentz LS, Woodside JV. Dietary patterns and breast cancer risk: A systematic review and meta-analysis. *American Journal of Clinical Nutrition.* 2010;91(5):1294-302.
29. Rastegari F, Rafeian-Kopaei M. Antioxidant supplements and cancer Correspondence to. *Immunopathol Persa.* 2016;2(2):e14. [cited 2019 Oct 15]. Available from: www.immunopathol.com
30. Hew CS, Khoo BY, Gam LH. The Anti-Cancer Property of Proteins Extracted from *Gynura procumbens* (Lour.) Merr. *PLoS One.* 2013;8(7):e68524.
31. Cragg GM, Newman DJ. Plants as a source of anti-cancer agents. *J Ethnopharmacol.* 2005;100(1-2):72-9. Aug 22 [cited 2019 Oct 15] Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16009521>
32. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell.* 2011;144(5):646-74. Available from: <http://dx.doi.org/10.1016/j.cell.2011.02.013>
33. Pollio A, DeNatale A, Appetiti E, Aliotta G, Touwaide A. Continuity and change in the Mediterranean medical tradition: *Ruta* spp. (rutaceae) in Hippocratic medicine and present practices. *J Ethnopharmacol.* 2008;116(3):469-82. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/18276094>
34. Franken NAP, Rodermond HM, Stap J, Haveman J, Van BC. Clonogenic assay of cells *in vitro*. *Nat Protoc.* 2006;1(5):2315-9. [cited 2019 Oct 15] Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17406473>
35. Munshi A, Hobbs M, Meyn RE. *In vitro* Measures of Chemosensitivity Clonogenic Cell Survival Assay. *Methods Mol Biol.* 2005;110:20-8.

Cite this article: Parrkar PN, Srinivas BK, Krishnappa DK, Jayarama S. *Zanthoxylum rhesta* Crude Protein has Promising Pro-apoptotic and Anti-angiogenic Properties. *Asian J Biol Life Sci.* 2020;9(3):334-41.