Published online in http://ijam.co.in ISSN No: 0976-5921

International Journal of Ayurvedic Medicine, Vol 15 (1), 2024; 117-121

In vitro cytotoxicity and apoptosis inducing effect of *Vallarai Kirutham* with *Rasa Parpam* in HeLa Cell Lines

Research Article

Ilakkiya Gandhi B[1*], Meenakshi Sundaram M[2]

1. PG Scholar, 2. Professor and HoD, Department of Kuzhandhai Maruthuvam,
National Institute of Siddha, Chennai, Tamilnadu. India.

Abstract

Cancer is an uncontrolled proliferation of cells that can affect nearby tissues as well as distant organs. Cervical cancer is predicted to be among the four most prevalent cancers in women overall in 2020, with 342,000 mortality and 604,000 new cases. Around 6-29% of all cancers in women in India are caused by cervical cancer. Women who have had several sexual partners, recurrent abortions, or vaginal deliveries all of which cause repetitive stress to the cervix are more likely to develop cervical cancer. Radiation, chemotherapy, and surgery are being used to treat cancer. All of these treatments come with side effects, which can range in severity and have a serious psychological impact on patients. The Siddha system has a number of accessible herbo-mineral formulations for treating cervical cancer. According to Siddha literature (*Agasthiyar Vaidhya Rathina Churukkam, 360),* the treatment of cervical cancer (*yoni puttru)* is advised for the Siddha medicine *Vallarai Kirutham* with *Rasa parpam*. The goal of this research was to find out this formulation's potential anti-cancer properties using MTT assays on HeLa cell lines. These findings of the current study suggest that the MTT technique's lowest reading for cell viability was 1.26±0.009% at a concentration of 100 µl/ml. This was followed by concentrations of 75 µl/ml, 50µl/ml and 25µl/ml which showed 7.91±0.004%, 23.91 ±0.01% and 37.17± 0.01% similarly, 10 µl/ml shows 52.09±0.01%.The matching IC50 value was discovered to be 12.44% and AO/EB dual staining was used to examine apoptotic activity. The outcomes of in-vitro experiments using the HeLa cell line. According to the outcome of in vitro experiments done on the HeLa cell line, the drug had substantial anti-cancer and therapeutic value in the management of cervical tumours. With a variety of time tested medications the Siddha system has shown outcomes that were clinically important for the management of cervical carcinoma. One of those medications has to undergo additional testing in patients with cervical cancer through clinical research.

Keywords: Cervical cancer, MTT assay, *Yoni puttru,* Anti -cancer activity, AO/EB dual staining, Chemotherapy, *Vallarai Nei*.

Introduction

Cancer is a collection of illnesses that are defined by unrestrained cell proliferation, which interferes with normal bodily functions and has serious implications for the patient. One in six women will develop cancer at some point in their lives, and one in eleven will pass away from it. The burden is growing at an incredible rate and is predicted to double by 2030. Patients with cancer experience significant psychosocial anguish. According to estimates, there will be 604,127 new cases and 342,831 fatalities from cervical tumours in women in 2020. In lower and mid economic regions during 2020, the majority of new cases and fatalities will occur (1)(2) 13,960 women in the United States are expected to have an invasive cervical cancer diagnosis in 2023.It is in the United states 4,310 fatalities from this illness are anticipated in 2023(3). However, the death rate for black women is 65% greater than for white women. Most cases of cervical cancer are discovered between the ages of 35 and 44. Persistent infection with high risk HPV strains is the primary cause of cervical cancer(4)

The predominant locations for female malignancies in most developing nations are the breast and cervix, which pose serious public health issues. Economic considerations, sexual behaviour and the effectiveness of mass screening are the main factors influencing the occurrence of cervical cancer in the community. Radiation, chemotherapy and surgery are some of the latest cancer treatments available. All of those procedures come with side effects which can have a serious psychological impact on patients. It is a special system that also upholds moral, mental and physical wellness. Due to their powerful anti oxidant action, certain Siddha medications are administered by qualified healthcare professionals for the treatment of cancer without causing any adverse effects.

In siddha literature, cancer is described using the word *puttru*, which convey its direct meaning. Cervical cancer is also referred to as *yoni puttru*. There are various simple formulas available to treat cervical

*** Corresponding Author:**
Ilakkiya Gandhi B
PG Scholar,
Department of Kuzhandhai Maruthuvam,
National Institute of Siddha,
Chennai, Tamilnadu. India.
Email Id: ilakkiyabsms.96@gmail.com

cancer. In this regard *yoni puttru* which is specified in *Agasthiyar vaithiya rathna churukkam-360(*5) is advised for the use of the medication *Vallarai kirutham* with *Rasa parpam*. Additionally, *Mega noi, Soolai, Sori,Sirangu, Nakku noi, Kiranthi, Pun, Puraigal and Neerarugal* have all been mentioned. The goal of this current research was to investigate these HeLa cell lines ability to resist cancer when treated with the Siddha formulation *Vallarai kirutham* with *Rasa parpam*.

Materials and Methods

Vallarai kirutham mentioned in "*Agasthiyar vaithiya rathna churukkam*" contains

- *Vallarai* juice (*Centella asiatica*)-1 padi(1 litre)
- *Ponnankanni* juice (*Alternanthra sessilis*)-1 *padi* (1 litre)
- *Poduthalai juice* (*Phyla nodifera*)-1 *padi*(1 litre)
- lemon juice-1/2 litre
- Cow ghee-2 litres
- Cow milk-2 litres
- *Jathikkai* (*Myristica fragrant*)
- *Jathipathiri, Milagu* (*Piper nigrum*)
- *Kirambu* (*Syzygium aromaticum*)
- *Athimathuram* (*Glycyrrhiza glabra*)
- *Maasikkai* (*Quercus infectoria*)
- *Karkadagasingi* (*Pistacia integerrima*) each 10 gms
- *Kattathi poo* (*Woodfordia fruiticosa*)
- *Elam* (*Elettaria cardamomum*)

Vallarai juice, *Ponnankanni* juice, *Poduthalai* juice are added to cow's ghee and cow's milk. Then *Jathikkai, Jathipathiri, Milagu, Kirambu, Athimathuram, Maasikkai, Karkadagasingi, Kattathi poo, Elam* are added and boiled .Then a little amount of sugar is added and the ghee is prepared. It is given *karandialavu* (16ml) with *Rasa parpam* for 48 days to treat *yoni puttru, kushtam, sori, sirangu, kiranthi*.

Rasa parpam mentioned in the siddha text "*Agasthiyar paripooranam 400*" (6)

Preparation: *Rasam* (Mercury quick silver) is grinded by *gandhaga dravagam* (Purified sulphur). Then take *Poovarasampattai* (*Thespesia populnea*)-2 *palam*(41gm) and *Nellikai gandhagam* -2 *palam* (41gms) is grinded with *siruthumbai* juice (*Leucus aspera*) and make it *kavasam* around the *rasam* and conduct *pudam* process.

Vallarai Kirutham and *Rasa parpam* are purchased from a pharmacy that has a GMP certification. All of the substances utilised in this investigation were analytic grade and got from a reputable laboratory. The alcoholic extracts were evaluated against these human HeLa cell lines for a percentage of viability among cells and apoptotic activities are investigated using the AO/EB dual staining method.

Cell line culture

It is possible to acquire Human cervical carcinoma cells from the National cell Science Centre for Cell Science in Pune, India. The cells were nurtured at 37°c in an incubator containing 5% of carbon dioxide employing DMEM (Dulbecco's Modified Eagle's Medium) besides 1% bacterium antimycotic solution (combination of penicillin and streptomycin) and 10% of blood from prenatal animals for 24 hours.

Cytotoxicity by MTT Procedure

In a 96-well plate without a test product, inoculate 200µl of the cell solution at the necessary cell density (Twenty thousand cells per well) and permit the cells to grow for 24 hours. Then add the testing reagents in the specified concentrations (10ul/ml, 50ul/ml, 75ul/ml, and 100ul/ml). The plate should be set at 37°c with 5% CO_2 for 24 hours. After the incubation period, remove these plates from the incubator. Thereafter adding MTT reagent to the entire volume at a concentration of 0.5 mg/ml. Shield the plate from light and enfold it in aluminium foil.

Place back the plates in the incubator and let them stay there for 3 hours. Empty the MTT reagent and add 100 ml DMSO lysis buffer**.** Gently stir after placing in a gyratory shaker to improve dissolution. Some of the time, particularly in thick culture pipetting up and down may be vital to altogether break up the MTT formazan crystals. Degree the absorbance at a wavelength of 570 nm employing a spectrophotometer.

Statistical analysis

The harmful chemical concentration at which biological activity is suppressed by 50% is known as the IC50 (Median Inhibition Concentration). With the MTT assay results as a starting point, for a non-linear regression IC50 is got in Microsoft Excel. The results will show statistical significance when it shows p value of $p < 0.01$ and $p < 0.05$.

Ethidium Bromide (EtBr) and Acridine Orange (AO) Double Staining for apoptosis determination:

Culturing the HeLa cells:

It is possible in order to purchase HeLa(Human cervical carcinoma cell line) from NCCS in India's Pune city. The cells had been grown in DMEM high glucose media loaded with 10% fetal bovine serum (FBS) and 1% bacterial antimycotic solution at 37°c within the CO_2 incubator under a 5% CO_2, 18-20% O_2 atmosphere. Every three days, the cells were subcultured. Study passage number 46 was used.

Principle

DNA binding dyes AO/EB(Acridine orange and Ethidium bromide) are used for apoptotic body formation and nuclear alterations associated with apoptosis. Intercalating acridine orange can penetrate both living and non-living cells. All nucleated cells are stained by AO, which creates green fluorescence. Ethidium bromide(EtBr), which displays red fluorescence and can be stimulated at 488nm, is the most commonly used.

Procedure

2×10^5 cells per ml of cultured cells should be used and the plate should be incubated in the CO_2 incubator at 37°c overnight. Cells should be incubated

for 24 hours after treating them with sample LD50 concentration of 12.44 L/ml and control. Harvest the cells into 2ml Eppendorf tubes at the conclusion of the therapy. Stain cells for 10 minutes in 200µl of staining solution. Remove the staining agent, then wash with PBS to get rid of the extra dye. Before imaging, carefully place 50 µl of the cell suspension onto the glass slide and mount it there by placing a drop of the mounting medium under the coverslip.

Use a filter cube and a fluorescence microscope to observe under conditions where EtBr is excited at 560/40nm and emitted at 645/75nm and acridine orange is excited at 470/40nm and emitted at 525/50nm. ImageJ software version 1.48 was used to overlay the images. Necrotic (cells uniformly orange-stained cell nuclei), late apoptotic (cells bright color green nuclei with condensed or fragmented chromatin), live (cells normal green nucleus), and early apoptotic (cells bright green nuclei with condensed or fragmented chromatin) were the four groups into which the cells had been separated.

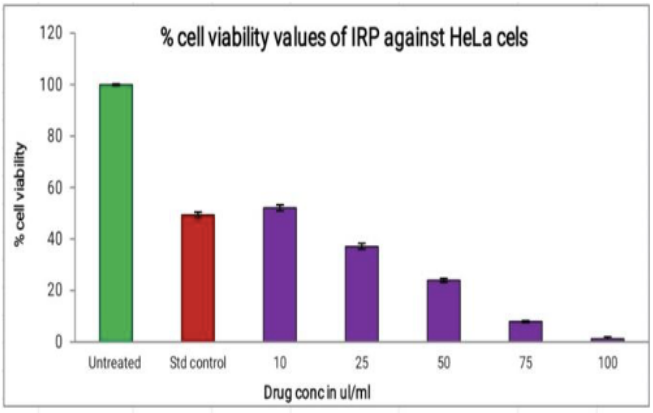
Results & Discussion

The test substance, *Vallarai kirutham* with *Rasa parpam*, has proven cytotoxic in nature on Human cervical cancer(HeLa) cells, according to the results of a cytotoxicity research carried out using MTT assay. Using the MTT test, this study was carried out at various concentration levels for estimating the IC50.

The results were summarized in fig.1 and depicted schematically shown in Graphs-1 and 2

Culture condition	%cell viability	IC50conc (ul/ml)
Untreated	100.00	
Std control	49.39	
IRP-10ul/ml	52.09	
IRP-25ul/ml	37.17	**12.44**
IRP-50ul/ml	23.91	
IRP-75ul/ml	7.91	
IRP-100ul/ml	1.26	

Graph 1: Shows the analyzed medications percentage of viability in a dose -dependent way.



Graph 2: Reveals the percentage of inhibition and the concentration of *Vallarai kirutham* with *Rasa parpam*

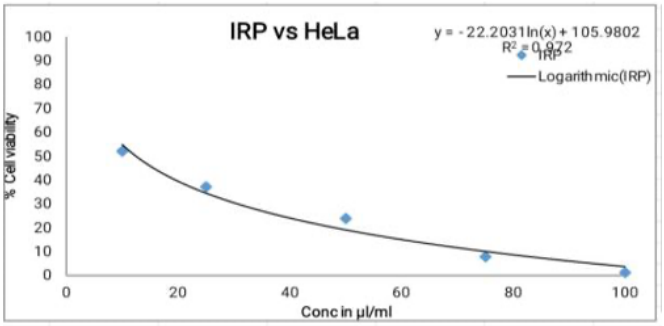
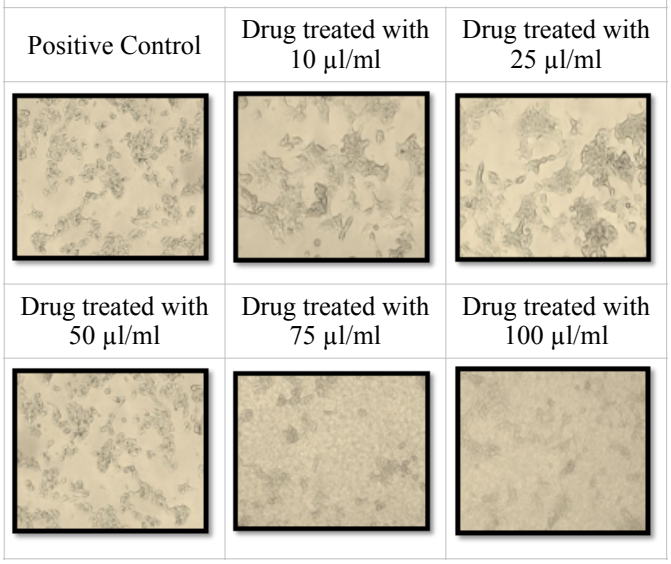


Figure 2: *Vallarai kirutham* with *Rasa parpam* at different dosage treated in HeLa cellline



After the treatment with *Vallarai kirutham* and *Rasa parpam* extract the result indicates the trial drug dose and the percentage of human HeLa cells that were inhibited. The trial medicine *Vallarai kirutham* with *Rasa parpam* was discovered to be inhibiting growth at a higher percentage at higher concentrations. The lowest cell viability was recorded at the concentration of 100µl/ml 1.26 ±0.009% and this was followed by concentrations of 75µl/ml,50 µl/ml, and 25 µl/ml showing 7.91±0.004%, 23.91±0.01%,37.17± 0.01 % similarly 10 µl/ml shows 52.09± 0.01% cell viability in MTT experiment. It was discovered that the equivalent IC50 value was 12.44%.

For 24 hours varied doses of *Vallarai kirutham* with *Rasa parpam*(10-100 µl/ml of 5%DMEM) were given. It was revealed that the amount of cells declines with dose and using an extract dosage of around 12.44 µl/ml, 50% of the cells(HeLa cells) were less than the usual control, as shown in Fig.1

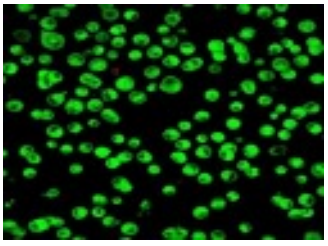
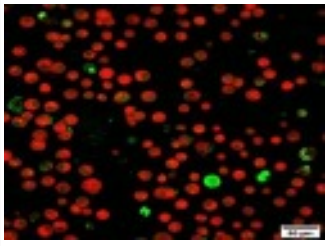
Our MTT results revealed that the combination of *Vallarai kirutham* and *Rasa parpam* considerably decreased the viability of the HeLa cell line. This finding is consistent with other research in which *Vallarai kirutham* was shown to have an anti-proliferative effect on cervical cell lines. This demonstrates the anti-cancerous properties of the Siddha formulation *Vallarai kirutham* with *Rasa*

parpam and the evidence suggests that the trial medicine can be a potential source of drugs for the treatment of cervical cancer.

AO/EB Fluorescent Assay for Cell Death:

IC 50 value concentration of *Vallarai kirutham* with *Rasa parpam* was applied to the HeLa cell line and incubated for 24 hours. The outcome reveals that more than 95% of the control cells are still alive, have evenly dispersed AO stain (green fluorescence), have normal nuclear morphology, and lack red fluorescence, which denotes apoptosis. Nearly 80% of the drug-treated cells have late apoptotic orange-stained nuclei with condensed or fragmented chromatin, 15% have uniformly necrotic orange-stained cell nuclei and 5% have early apoptotic bright green nuclei with condensed or fragmented chromatin.

Fig-2 The presence of an AO/EB stain on HeLa cells subjected to control and drug treatment(*Vallarai kirutham* with *Rasaparpam*)

Control	Treated with drug (VK and RP)
	

The staining of AO/EB following drug treatment reveals the onset of early apoptosis with bright green with condensed or broken chromatin and demonstrates necrotic changes with an Orange stained nuclei in comparison to control HeLa cells.

According to the results of cell counting, the medications cause more than 95% of the cells to undergo apoptosis, which suggests that they may have anti cervical cancer properties. After AO/ EB dual-labeling the morphological characteristics of apoptosis were further examined by viewing under a fluorescence microscope. Most of the chemotherapeutic medicines used to treat cancer cause apoptosis, a gene-regulated process that causes DNA fragmentation, membrane blebbing, nuclear shrinkage and chromatin condensation(7, 8)

Saponins have a wide range of complex structural variations and have demonstrated significant anticancer activity in a number of cancer cell lines by preventing cell proliferation and triggering apoptosis(10)

The drug's capacity to induce apoptosis was anticipated because one of its active ingredient, *Centella aciatica* (L.) Urban contains saponin(9)

Saponins effect on cytotoxicity. Saponins exhibit lethal effects on cancer cells in addition to their chemopreventive properties.

Treatment with saponin can promote autophagic cell death in cancer cells. (11)

The function of saponin in cancer and its mechanisms of action, including cell cycle arrest, and the antioxidant activity of autophagy have been the subject of several research(11)

Phytochemical investigation in *Vallarai kirutham* revealed the presence of phenols and terpenoids, according to a preliminary study. Phenols can improve the body's immune system's ability to detect and kill cancer cells as well as reduce angiogenesis, the process of making new blood vessels required for the growth of tumors (12)

According to Linhua Quin et al, cervical cancer cells undergo cytotoxicity and apoptosis when AuNPs are derived from *Alternanthera sessilis* (L.) R.Br. (13). Tripathi nagja et al.The prophylactic properties of *Myristica fragrans*. Houtt. investigated the carcinogenesis caused by methylchol anthrene in the uterine cervix of Swiss albino mice.(14) Previous studies, *Piper nigrum* L. demonstrated anti-cancer properties against cervical cancer cell lines by a variety of processes, such as autophagy, apoptosis, and cytotoxicity (15). Ethyl acetate extract of clove was found to inhibit tumour growth and increase apoptosis and cell cycle arrest in a study by Liu H et al (16).Extracts of various components (gall, leaf, bark) of *Pistacia integerrima*. J.L.Stewart were carried out it revealed significant cytotoxic effect against the HeLa and BHK-21 cell line. Wajeeha Rani et al.(17)

Woodfordin C, a macro ring hydrolysable tannin dimmer from dried *Woodfordia floribunda salisb* (L.) Kurz flowers, has been found in recent investigations and is said to have anti-tumour properties. Yosida T et al. 1990 (18).

Deepa et al. conclude that the Siddha formulation Rasa parpam has possess anti-cancerous effect by MTT assay(19)

Conclusion

One of the most common types of cancer in women worldwide is cervical cancer; In contrast to industrialised nations, women in underdeveloped nations are major cervical victims. The current cancer treatments include hysterectomy, chemotherapy, radiotherapy, photodynamic therapy, and catalytic therapy all of which have a number of negative effects. Alternative medications are many. One of these is the Siddha system of medicine. This medical system has identified a number of drug formulations that make claims regarding the management and treatment of numerous diseases, including cancer. Siddha medicines are very safe and have effective therapeutic results without any negative side effects. To conclude the Siddha drug *Vallarai kirutham* with *Rasa parpam* more effectively induces apoptosis and inhibits cell growth in the HeLa cell line.

References

1. Singh D, Vignat J, Lorenzoni V, et al. Global estimates of incidence and mortality of cervical cancer in 2020: a baseline analysis of the WHO Global Cervical Cancer Elimination Initiative. Lancet Glob Health. 2023;11(2):e197-e206. doi:10.1016/S2214-109X(22)00501-0

2. Hull R, Mbele M, Makhafola T,et al. Cervical cancer in low and middle-income countries. Oncol Lett. 2020;20(3):2058-2074. doi:10.3892/ol.2020.11754
3. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics.CA Cancer J Clin. 2023;73(1):17-48. doi:10.3322/caac.21763
4. Kombe Kombe AJ, Li B, Zahid A, et al. Epidemiology and Burden of Human Papillomavirus and Related Diseases, Molecular Pathogenesis, and Vaccine Evaluation. Front Public Health. Jan .2021;8:552028. doi:10.3389/fpubh.2020.552028
5. Thirumalaichamy R.M.K. Agasthiyar Vaidhya Rathina Churukkam- 360.Moolamum uraiyum.Chennai: K. A. Madurai muthaliyar.1937.pp-207
6. Vadivelu Mudhaliyar M.Agasthiyar paripooranam-400. Suthapathippu. Chennai. Ganesha printing press, 1921. Page no-78
7. Wlodkowic D,Skommer J,Darzynkiewicz Z.Flow cytometry-based apoptosis detection. Methods Mol Biol. 2009;559:19-32. doi:10.1007/978-1-60327-017-5_2
8. Yakovlev AG, Faden AI. Mechanisms of neural cell death: implications for development of neuroprotective treatment strategies. NeuroRx. 2004;1(1):5-16. doi:10.1602/neurorx.1.1.5
9. Gray NE, Alcazar Magana A, Lak P, et al. *Centella asiatica* - Phytochemistry and mechanisms of neuroprotection and cognitive enhancement. Phytochem Rev.2018;17(1):161-194. doi:10.1007/s11101-017-9528-y.
10. Xu XH, Li T, Fong CM, et al. Saponins from Chinese Medicines as Anticancer Agents. Molecules. 2016;21(10):1326. doi:10.3390/molecules21101326
11. Elekofehinti OO, Iwaloye O, Olawale F, Ariyo EO. Saponins in Cancer Treatment: Current Progress and Future Prospects. Pathophysiology. 2021; 28(2): 250-272.
12. Ravichandran M, Evaluation of phytochemical and in vitro anti-oxidant, anti proliferative activity of a polyherbal Siddha formulation Vallarai nei, Int.J.Curr.Microbiol.App.Sci, 2014 ; Volume 3(4): 161-171
13. Qian L, Su W, Wang Y, Dang M, Zhang W, Wang C. Synthesis and characterization of gold nanoparticles from aqueous leaf extract of *Alternanthera sessilis* and its anticancer activity on cervical cancer cells (HeLa). Artif Cells Nanomed Biotechnol. Dec 2019;47(1):1173-1180. doi:10.1080/21691401.2018.1549064
14. Tripathi Nagja.K,Vimal et al,*Myristica fragnans*:A Comprehensive review,International journal of Pharmacy and Pharmaceutical Sciences , Feb 2016. Vol 8, Issue 2. pp. 27-30,
15. Takooree H, Aumeeruddy MZ, Rengasamy KRR, et al. A systematic review on black pepper *(Piper nigrum* L.): from folk uses to pharmacological applications. Crit Rev Food Sci Nutr. Feb 2019;59(sup1):S210-S243. doi:10.1080/10408398.2019.1565489
16. Liu H, Schmitz JC, Wei J, et al. Clove extract inhibits tumor growth and promotes cell cycle arrest and apoptosis. Oncol Res. 2014;21(5):247-259. doi:10.3727/096504014X13946388748910
17. Rani W, Maqbool F, Bhatti ZA, et al. Antibacterial and Anticancer Efficacy of Different Parts of *Pistacia Integerrima* Plant Extracts. Research Square;April 2021. DOI: 10.21203/rs.3.rs-396639/v1.
18. Yoshida T, Chou T, Nitta A, Miyamoto K, Koshiura R, Okuda T. Woodfordin C, a macro-ring hydrolyzable tannin dimer with antitumor activity, and accompanying dimers from *Woodfordia fruticosa* flowers. Chem Pharm Bull (Tokyo). 1990;38(5):1211-1217. doi:10.1248/cpb.38.1211
19. Deepa.G,Velpandipan, Scientific validation of siddha formulation Rasa parpam and its anticancer property in hela cell line an invivo and invitro assay, World Journal of Pharmaceutical Research, published on Sep 2019 Volume 8, Issue 11, 1105-1132.

*****