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IN VITRO ANTI-TUMOUR EFFECTS OF DIETARY PHYTOESTROGENS

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ABSTRACT

Diet and nutrition are known to contribute to different rates of cancer prevention/progression. Estrogens are sex hormones which exert many biological and physiological functions in humans. Deficiency of estrogens postmenopause, result in multifaceted complications in women including increased risk for the development of cardiovascular diseases and osteoporosis. The gold standard treatment for osteoporosis in postmenopausal women is Hormone replacement therapy with synthetic estrogens. The major limitations of estrogen therapy is the potential proproliferative effects of estrogen on reproductive organs like breast and uterus that predisposes the recipient to high risk of developing breast and endometrial cancers. But these side effects could be avoided with the use of phytoestrogens, as many of them are selective estrogen receptor modulators exhibiting tissue selectivity. Biochanin A, Formononetin and Resveratrol are phytoestrogens which mimic the actions of endogenous estrogens but are devoid of the side effects of synthetic estrogen. Although a plethora of scientific reports are available to support the osteoprotective effects of these phytoestrogens, scientific reports pertaining to their anti-tumour property is scanty. Therefore it is desirable to study the antitumour properties of these phytoestrogens on suitable *in vitro* or *in vivo* model systems. This would justify and provide a scientific rationale for its probable use as a safe potential alternative to Hormone Replacement Therapy with estrogen. This would also help to understand the anticarcinogenic/procarcinogenic effects of these compounds thereby throwing light on their mechanisms of action. This preliminary study is an attempt to

understand the *in vitro* antitumour properties of three important dietary phytoestrogens Biochanin A, Formononetin and Resveratrol.

Keywords: Formononetin, Biochanin A, Resveratrol, Phytoestrogens, *Agrobacterium tumefaciens.* Phytopathogenicity tests

1. INTRODUCTION

Formononetin, biochanin A and resveratrol are estrogenic compounds produced as a defensive response by many plants to fight against pathogens. Having structural similarity with endogenous estrogens, these compounds can bind to estrogen receptors and influence the downstream signaling events controlling many physiological processes in humans. All the three phytoestrogens have a higher affinity for estrogen receptor beta thereby the compounds could be devoid of the proproliferative effects on organs like breast wherein the estrogen receptors are predominantly the alpha type. This selective affinity towards estrogen receptor beta enable them to act as Selective Estrogen Receptor Modulators (SERMS) which could possess estrogen receptor agonistic or antagonistic effects based on target tissue. SERMs could be potential antitumour and antiproliferative agents. This property of SERMs like formononetin, biochanin A and resveratrol project them as safe alternatives to synthetic estrogen which is frequently used in Hormone replacement therapy for the management of many diseases. The current study is an attempt to understand the antitumour properties of these three phytoestrogens through simple bioassays which could provide preliminary idea about their role as effective anticancer agents.

Phytoestrogen therapy has been shown to inhibit inflammation, angiogenesis and metastases in various *in vivo* tumor models, and pronounced benefits observed when combined with radiation therapy **[1]**. A number of studies have described an important role of phytoestrogens in regulating the cell cycle. In a highly metastatic bladder cancer cell line (253J B-V), genistein has been shown to inhibit cell growth by inducing cell cycle arrest at the G2/M transition, and significantly reduced the expression of cell cycle regulators cyclin B1 and Cdk-1 **[2]** . Formononetin also possesses hypolipidemic properties, mammary gland proliferation function, and antitumor effect in colon and breast cancer **[3].** Estrogenic effect of formononetin has also been reported. This isoflavone has structural similarity with 17-estradiol; thus, it can bind to estrogen receptors, for example, in bone tissue **[4,5,6]**.

Biochanin A is the main isoflavone component of red clover (*Trifolium pratense* L). In contrast to its unmethylated

analogue genistein, biochanin A is not present in soy at significant quantities, but it can be found in many other legume plants and peanuts. The role of biochanin A and other isoflavones in these plants is not known in detail, but generally speaking such secondary metabolites are produced to protect the plant from radiation and microbial attacks. Most beneficial health effects linked to isoflavones such as Biochanin A are believed to be mediated by the estrogenic and antioxidative properties of these compounds. Epidemiological studies have demonstrated the protective effects of isoflavone-rich diets against breast and prostate cancer **[7]**, and the affinity of isoflavones to estrogen receptors is thought to mediate also their osteoprotective effects **[8]**. Although estrogenic soy isoflavones like genistein and daidzein has been extensively investigated for their biological actions, the other dietary isoflavones like Biochanin A and Formononetin have not been studied in detail especially with regard to their antitumour properties **[9].** The role of Resveratrol as a potent antioxidant and cardioprotectant is well established but investigations with regard to its antitumour properties are relatively less.

Hence the objective of the present study is i) To assess the anti-tumour properties of the three phytoestrogens biochanin A, formononetin and resveratrol against *Agrobacterium tumifaciens*-induced tumours *in vitro* (disc based bioassay). ii) To assess the growth inhibitory effects of the phytoestrogens against *Agrobacterium tumifaciens* culture.

2. MATERIALS AND METHODS

2.1. Procurement and maintenance of *Agrobacterium tumifaciens* culture: *Agrobacterium tumifaciens* (strain EHA 105*)* culture was kindly provided by **Dr. Manmohan,** Principal Scientist**,** Division of Biotechnology, Indian Institute of Horticultural Research (IIHR), Hesarghetta, Bangalore. The organisms were cultured on Luria bertani media (in LB Agar/broth as required for the assay). The growth media was prepared using the following composition :10g of yeast extract, 10g of peptone, 5g of Nacl, 20g of agar dissolved in 1 litre of water. The stock cultures (broth cultures of *Agrobacterium tumifaciens*) were inoculated on LB agar plates and cultured at 25℃ for 48hrs. After 48 hours, a single colony from the plate was transferred into LB broth and the cultures were allowed to grow at 25℃ for 48hrs. Suspensions of freshly grown *Agrobacterium tumifaciens* cultures were used for the experiments at cell numbers as required for the respective assays.

2.2. Prepration of stock solutions of Phytoestrogens: A stock solution of 1 mg/ ml of the test compounds (Biochanin A, Formononetin and Resveratrol was

prepared in DMSO (the final concentration of DMSO when used in culture was less than 0.1%) and stored at -20°C. From the stock solution different concentrations of the test compounds (appropriately diluted) was prepared and used for different assays.

2.3. ASSAYS PERFORMED

2.3.1 Determination of minimum inhibitory concentration by microdilution method:

This was performed following the method described by Mosmann 1983 **[10].** Briefly the cells (100 µl of *Agrobacterium* cultures adjusted to a concentration of 10^7 cells/ml) were plated on to the wells of a 96 well microtiter plate. 50 µl of different concentrations of Formononetin, Biochanin and Resveratrol (125, 250, 500 and 750µg/ml) were then added to the wells and the cells were allowed to grow for 48 hours. After 48 hours, 30µl of MTT (5mg|ml in phosphate buffered saline -pH 7.4) was added to the wells. The plates were incubated for 2 hours. After 2 hours the formazan crystals produced were solubilized by adding 70µl of DMSO to each well and the intensity of the colour developed was read at 570 nm in a micro plate reader (Biorad, India). A graph was plotted with concentration of the compounds on the X axis and the absorbance on the y axis and the minimum concentration of the test compounds which inhibited the growth of *Agrobacterium tumefaciens* was determined.

2.3.2 Agar well diffusion assay:

Agar well diffusion assay was performed by following the method of Kalemba and Kunicka, 2003 **[11].** Briefly, LB agar was prepared, sterilized, poured into sterile petri plates and allowed to solidify. 200µl of broth cultures of *Agrobacterium tumifaciens* was inoculated on to the LB agar plates by spread plate method using a sterile glass spreader. Equally spaced wells were made on the agar plates using a sterile cork borer. 20µl of test compound (Biochanin A, Formononetin or Resveratrol) of different concentrations (500 and 750µg/ml) was added to each well. Wells containing DMSO was included as vehicle control wells. The plates were kept in an incubator at 25°C for 48 hours. After 48 hours, the plates were examined for the clearance zones around the wells and the diameter of the zone of complete inhibition was measured (to the nearest whole millimeter) with a ruler.

2.3. 3. Disc based bioassay for the assessment of anti-tumour activity (Phytopathogenicity tests on carrot, beet and potato discs)

Phytopathogenicity tests were done using carrot, beet and potato discs. The specified strain of *Agrobacterium tumefaciens* was used for the tumor induction . The tests were performed following the method of

antitumour disc assay described by Trigui et al., 2013 **[12]**. Briefly, *A tumefaciens* strains were cultured on Luria Bertani (LB)agar medium. A single colony was transfered into 25ml of LB broth and incubated at 25°c for 24 hours. Beets (*Beta vulgaris* L.), Carrots (*Daucas carota* L.) Potatoes (*Solanum tuberosum* L.) were washed by scrubbing under running water with a brush, then disinfected by using 70% isopropanol and immersed in 10% sodium hypochlorite solution for 10 minutes. After 10 minutes they were soaked in autoclaved distilled water for 10 -20 minutes. Potato, carrot and beet discs (5mm×8mm) were made with a sterile cork borer and rinsed with autoclaved water, blotted to dryness on sterile paper towels. 12 discs were placed on petriplates containing autoclaved agar medium(1.5%). Suspensions of *A.tumefaciens* on LB broth medium were standardised to 10^7 CFU/ml as determined by an absorbance value of 1.0 at 600 nm. Each disc was overlaid with 50µl of bacterial suspension following which the test compounds were added to the discs at different concentrations. Positive control discs containing the anticancer compound fisetin and negative control discs containing the carcinogen anthracene were put up along with the discs containing the phytoestrogens. The petriplates were then sealed using a parafilm and the plates were incubated at 25°C for 14-21days. Two replications were used and experiment was repeated at least twice.

Carrot discs were checked after 10 days for young galls developing from meristematic tissue around vascular system. Beet discs were also checked after 10 days for the development of tumors on the entire disc surface. The plates were examined under a dissection microscope and the number of tumors present in the control and test were counted. After 21 days, potato discs were stained with Lugol's solution (10% Potassium iodide and 5% Iodine). Lugol's reagent stains the starch present in the potato tissue a dark blue to dark brown colour, but the tumors produced by *A. tumefaciens* will not take up the stain, and appear creamy to orange.

Numbers of tumors per disc in all the three types of discs (carrot, beet and potato discs) were counted and percent inhibition for each concentration of the test compound was determined by the formula given below

$$\text{Percent inhibition of tumours} = 100\left(1 - \frac{\text{Mean no of tumours in the test sample}}{\text{Mean no of tumours in the control}}\right) \times 100$$

3. RESULTS AND DISCUSSION:

Formononetin, biochanin A and resveratrol are estrogenic compounds produced as a defensive response by many plants to fight against pathogens. Having structural similarity with endogenous estrogens, these compounds can bind to estrogen receptors and influence the downstream signaling

events controlling many physiological processes in humans. All the three phytoestrogens have a higher affinity for estrogen receptor beta thereby the compounds could be devoid of the proproliferative effects on organs like breast wherein the estrogen receptors are predominantly the alpha type. This selective affinity enable them to act as Selective Estrogen Receptor Modulators (SERMS) which could possess estrogen receptor agonistic or antagonistic effects based on target tissue. SERMs could be potential antitumour and antiproliferative agents. This property of SERMs like formononetin, biochanin A and resveratrol project them as safe alternatives to synthetic estrogen **[13]** (Martinkovich et al., 2014). The current study is an attempt to understand the antitumour properties of these three phytoestrogens through simple bioassays which could provide preliminary idea about their role as effective anticancer agents.

3.1. Effect of formononetin, biochanin A and resveratrol on the growth of *Agrobacterium tumifaciens*- MTT assay

MTT assay is a fast and reliable method in order to determine the growth inhibitory, cytotoxic and cytostatic effects of test compounds **[14]** (Florento et al., 2012). In the current study, MTT assay was performed to check the growth inhibitory effects of the compounds formononetin, biochanin A and resveratrol against actively growing *Agrobacterium tumifaciens* culture. Figure 3.1a, 3.1b and 3.1c shows the results of MTT assay performed on *Agrobacterium tumifaciens* strain EHA 105. The results revealed that all the three compounds inhibited the growth of *A.tumifaciens* with the best inhibition obtained with formononetin followed by resveratrol and biochanin A. Formononetin and resveratrol was effective in inhibiting the growth of *A.tumifaciens* at all the four concentrations studied (125, 250, 500 and 750µg/ml). Biochanin A was found to be ineffective in inhibiting the growth at the lowest concentration studied whereas high concentrations inhibited the growth of the bacterium effectively. At the highest concentration used in the assay (750µg/ml) almost 50% reduction in the cell population was observed. The MIC for formononetin and resveratrol was found to be 125µg/ml whereas it was found to be 250µg/ml for biochanin A. The growth inhibitory effects of the compounds were in the order of Formononetin > Resveratrol > Biochanin A.

3.2 Effect of formononetin, biochanin A and resveratrol on the growth of *Agrobacterium tumifaciens* - Agar well diffusion method.

Agar well-diffusion testing is a routinely used method in many clinical microbiology laboratories for antimicrobial susceptibility

testing **[15]**. In the current study, agar well diffusion test was performed to check the results of MTT assay wherein effective inhibition in growth was observed against *A. tumifaciens* culture. Figure 3.2a and 3.2b shows the results of agar well diffusion assay with formononetin, 3.2c and 3.2d shows the results with biochanin A and 3.2e and 3.2f shows the results with resveratrol respectively. As observed in MTT assay, effective inhibition of growth of *A.tumifaciens* indicated by clearance zones around the test well was seen with all the three compounds with the best growth inhibitory effects and maximum zone of inhibition obtained with formononetin. The zones of inhibition was found to be 1 cm and 1.4 cm for formononetin; 0.4cm and 1.1 cm for biochanin A and 0.8 and 1.4cm for resveratrol. Both the concentrations used in the assay (500 and 750µg/ml) was found to be effective in inhibiting the growth.

3.3. Effect of formononetin, biochanin A and resveratrol inhibiting crown gall tumours- Carrot, beet and potato disc based bioassays

Bioassay offers special advantages to know about the biological activity of plant extracts and

provide information to isolate active compounds which is a preliminary key step for drug discovery system. Several bioassays such as brine shrimp cytotoxicity assay, radish seed phytotoxicity assay, lettuce seedling growth assay, and the potato disc bioassay have been developed for biological screening of medicinal plants. *Agrobacterium tumefaciens* is a soil borne Gram-negative bacterium which has a unique type of Ti plasmid containing T-DNA region. By transferring T-DNA region into plant cell through type IV secretion system *A. tumefaciens* produces crown gall disease in plants. Crown gall is a neoplastic diseases of plants which occur in more than 60 families of dicots and many gymnosperms. The disease is characterized by the transformation of normal plants cells into autonomous tumor cells in a short period of time. The causative agent for this disease are specific strains of gram negative bacterium *Agrobacterium tumefaciens.*

During infection of plant material with *A.tumefaciens*, a tumour producing plasmid (Ti plasmid) is incorporated into the plant chromosomal DNA. When plant tissue is wounded it releases phenols which will activate the Ti- plasmid in *A. tumefaciens*. The Ti- plasmid causes the plant cells to multiply rapidly without going through apoptosis resulting in tumour formation similar in nucleic acid content and histology to human and animal cancers. The rationale for use of bioassay is that the tumorigenic mechanism initiated in plant tissue by *Agrobacterium tumefaciens* is

similar to that of animals; the relevance of crown gall tumour system to the general cancer problem has been thoroughly understood. Crown gall tumours could be routinely used as a comparative, rapid, safe and inexpensive, and statistically reliable prescreen for *in vivo* antitumor activity **[16]**.

In the current investigation, the antitumour activity of the phytoestrogens Biochanin A, Formononetin and Resveratrol was checked using simple, easy to perform, inexpensive but reliable and reproducible assays like carrot disc assay, beet disc assay and potato disc bioassay. Two different concentrations of the compounds (750 and 500µg/ml of the test compounds)showed considerable inhibition in the number of tumours induced by *A. tumifaciens* in all the three disc based bioassays. Figure 3.3.1 shows the results of the carrot disc assay with formononetin and resveratrol (3.3.1.a-control, 3.3.1.b -FMN treated and 3.3.1.c RSV treated), 3.3.2 shows the results of beet disc assay(3.3.2.a-control, 3.3.2.b-FMN treated and 3.3.2.c RSV treated) 3.3.3 shows the results of potato disc assay (3.3.3.a- control, 3.3.3.b-FMN treated and 3.3.3.c- RSV treated) performed to evaluate antitumour activities of the compounds. In accordance with the results obtained in MTT assay and agar well diffusion assay, disc based bioassays also indicate the effectivness of the formononetin and biochanin A in inhibiting the growth of the crown gall tumour causing organism A. tumifaciens. Multiple tumours were observed in the discs containing *A.tumifaciens* culture. In the carrot based disc assay the percentage of tumour inhibition by formononetin was found to be 33% and by resveratrol was found to be 16%. Based on the beet disc bio assay, the percentage of tumour inhibition by formononetin was found to be 63% and 19% by resveratrol. Based on the results obtained with potato disc bio assay the percentage inhibition of tumour was found to be 60% with formononetin and 33% with resveratrol. no inhibition of tumour growth was observed in the discs treated with biochanin A thereby suggesting that the effect of biochanin A on the growth of *A.tumifaciens* could be cytostatic rather than cytotoxic. The antitumour activity of the compounds was found to be of the order Formononetin > resveratrol > biochanin A. Among all the three disc bioassays the best results were obtained in the beet disc assay for formononetin whereas resveratrol exhibited good antitumour properties in the potato disc bioassay. The results obtained indicate the potent antitumour properties of formononetin.

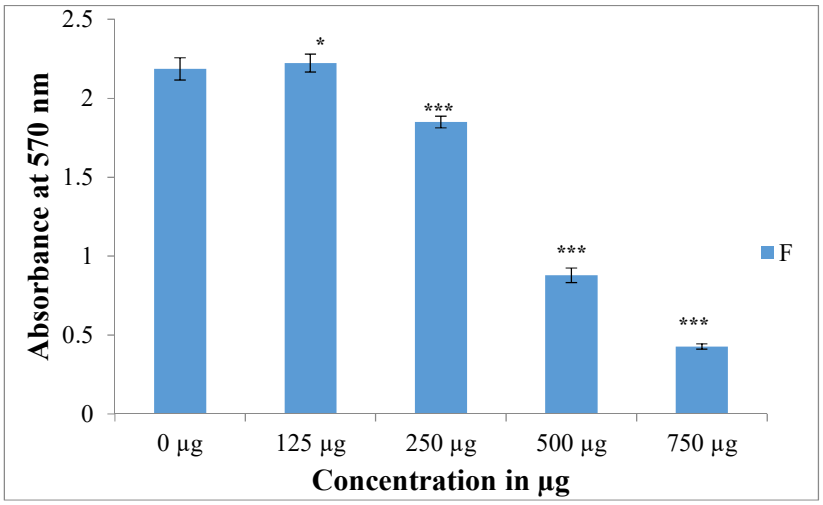


Figure 3.1a: Effect of Formononetin on agrobacterium growth (MTT assay).
Figure shows the effect of Formononetin on agrobacterium growth (MTT assay).Values were expressed as mean ± SD (n = 6). Comparisons were made between control Vs treated groups. * *p <0.001, *p<0.05***

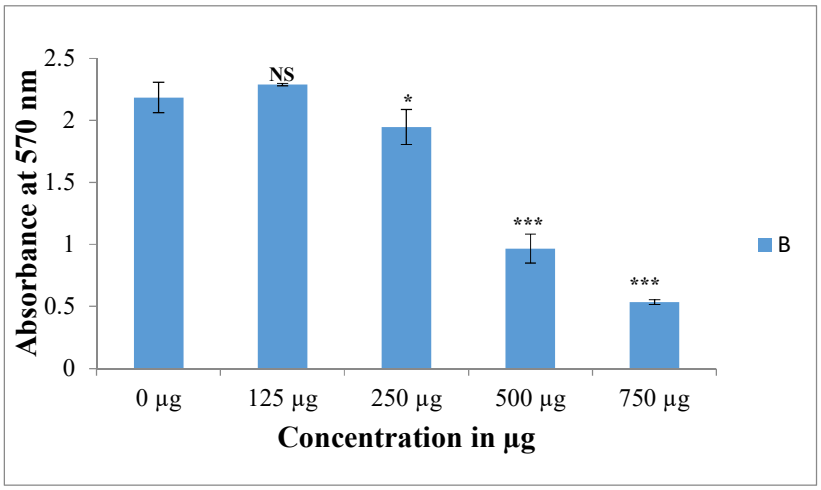


Figure 3.1b: Effect of Biochanin A on agrobacterium growth (MTT assay).
Figure shows the effect of Biochanin A on agrobacterium growth (MTT assay).Values were expressed as mean ± SD (n = 6). Comparisons were made between control Vs treated groups. * *p <0.001, *p<0.05***

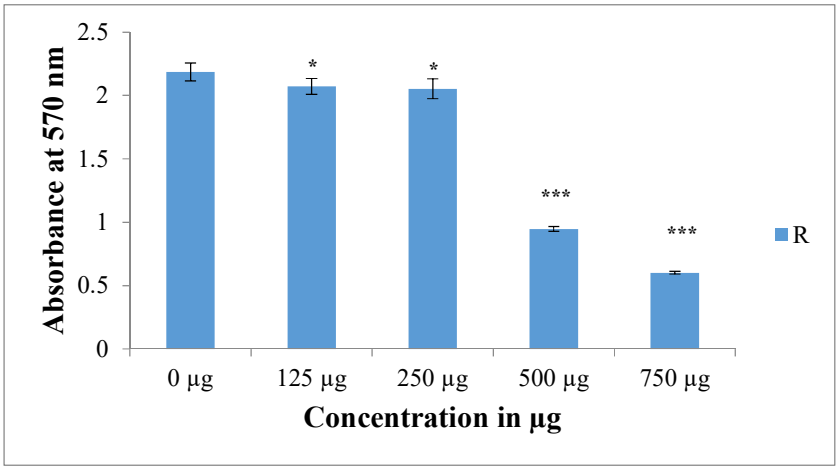
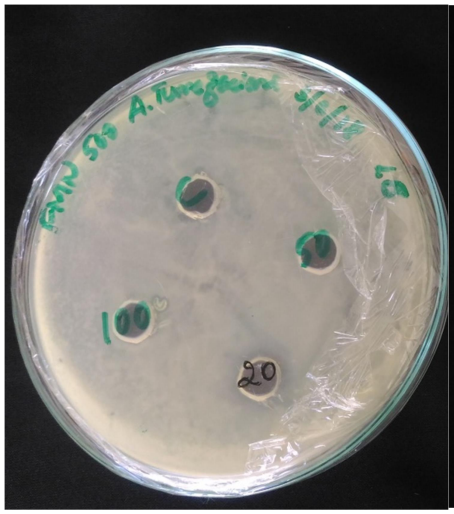


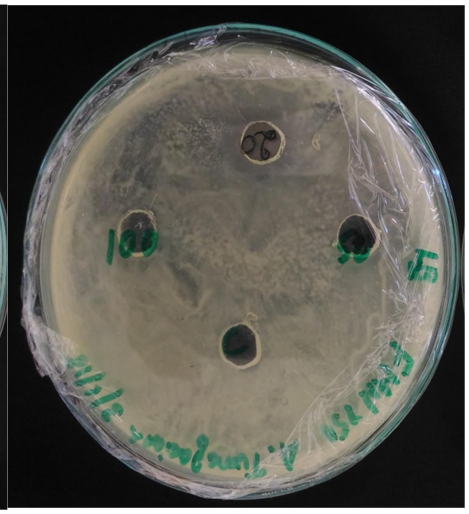
Figure 3.1c: Effect of Resveretrol on agrobacterium growth (MTT assay)
Figure shows the effect of Resveretrol on agrobacterium growth (MTT assay).Values were expressed as mean ± SD (n = 6). Comparisons were made between control Vs treated groups. * *p <0.001, *p<0.05***

Assessment of antimicrobial activity of phytoestrogens by agar well diffusion method



Agrobacterium Zone of inhibition 500 µg

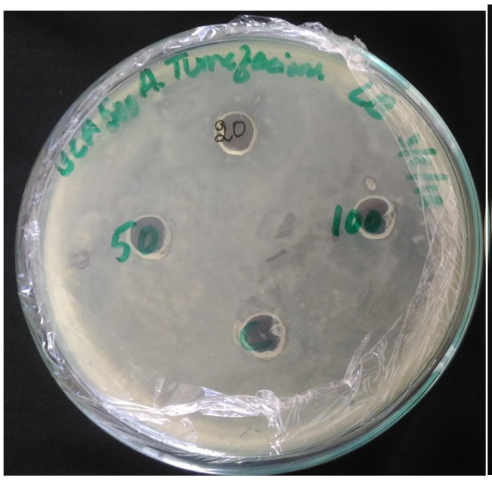
Figure 3.2.a



Agrobacterium Zone of inhibition 750 µg

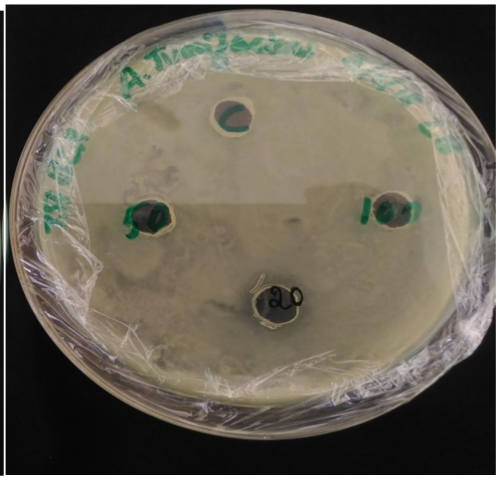
Figure 3.2.b

Antimicrobial activity of Formononetin on growth of *Agrobacterium tumefaciens*



Agrobacterium Zone of inhibition 500 µg

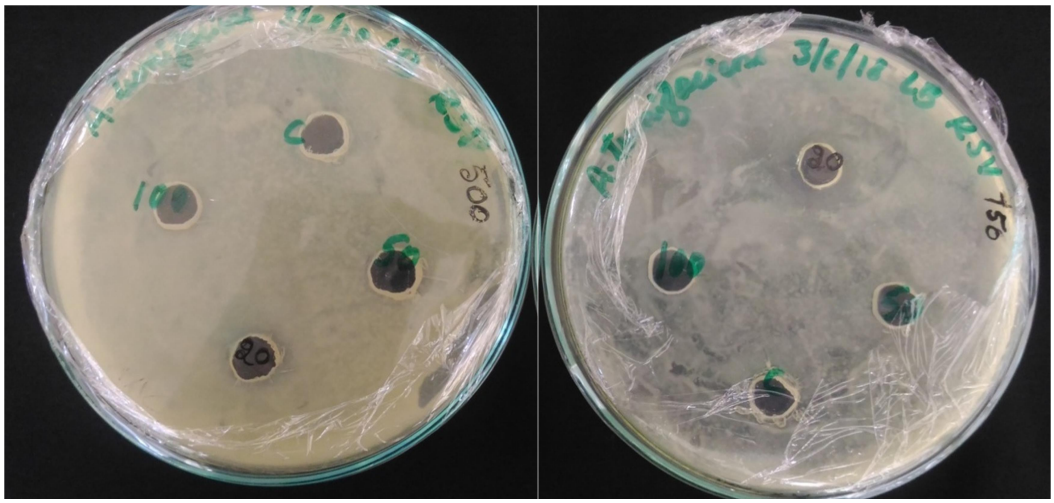
Figure 3.2.c



Agrobacterium Zone of inhibition 750 µg

Figure 3.2.d

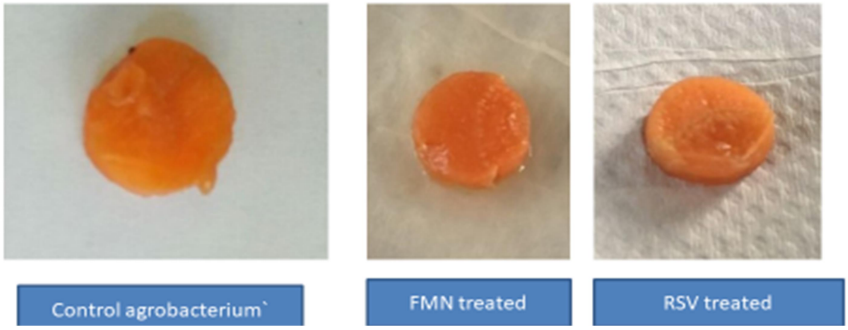
Antimicrobial activity of Biochanin A on growth of *Agrobacterium tumefaciens*



Agrobacterium Zone of inhibition 500 µg **Agrobacterium Zone of inhibition 750 µg**

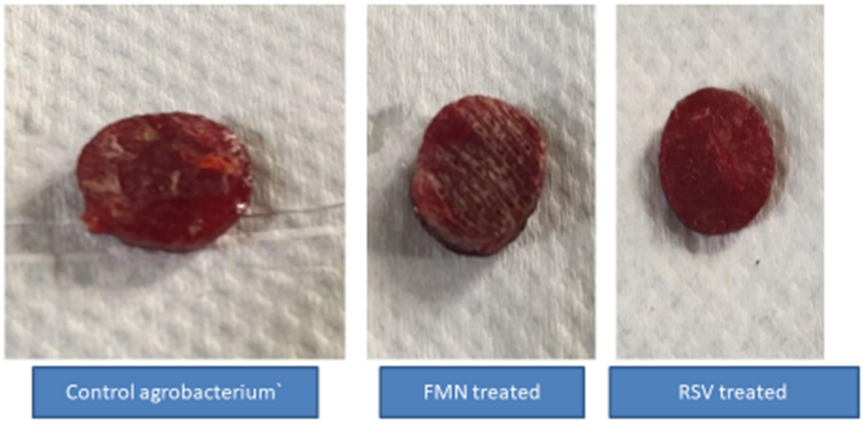
Figure 3.2. e **Figure 3..2.f**

(A) **Antimicrobial activity of Resveretrol on growth of *Agrobacterium tumefaciens***



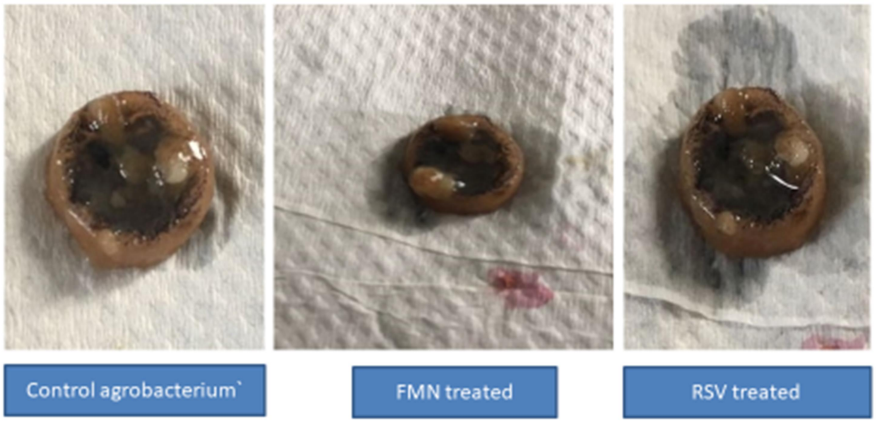
3.3.1.a **3.3.1.b** **3.3.1.c**

Figure 3.3.1- Results of phytopathogenicity test with formononetin and resveratrol on carrot discs



3.3.2.a **3.3.2.b** **3.3.2.c**

Figure 3.3.2- Results of phytopathogenicity test with formononetin and resveratrol on beet discs



3.3.3.a 3.3.3.b 3.3.3.c

Figure 3.3.3- Results of phytopathogenicity test with formononetin and resveratrol on Potato discs

4. CONCLUSION:

Overall the results of the current study implicate the potent anti-tumour effects of the phytoestrogens formononetin and resveratrol. Biochanin A was not as effective as the other phytoestrogens with respect to antitumour properties. The strong antitumour properties of these phytoestrogens opens up promising avenues for their probable use as safe alternatives for HRT in many pathological conditions like osteoporosis and hormone responsive breast cancers.

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