CHEMOPREVENTIVE EFFICACY OF *Vitis vinifera* GOLD NANOPARTICLES IN SKIN CARCINOGENESIS: AN *in vitro* AND *in vivo* APPROACH

*a thesis submitted by*

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in partial fulfillment for the award of the degree of

DOCTOR OF PHILOSOPHY

*under the supervision of*

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DECLARATION

I, J. GRACE NIRMALA hereby declare that the thesis, entitled “Chemopreventive efficacy of Vitis vinifera gold nanoparticles in skin carcinogenesis: an in vitro and in vivo approach”, submitted to the Karunya University, in partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy in Biotechnology is a record of original and independent research work done by me during the period 2009 – 2015, under the supervision and guidance of Dr. R.T. Narendhirakannan, Assistant Professor (SG), Department of Biotechnology, School of Biotechnology and Health Sciences, Karunya University. The work contained in this thesis has not been previously submitted to meet the requirements for a degree or diploma at this or any other higher education institution.

Signature of the candidate

(J. Grace Nirmala)
BONAFIDE CERTIFICATE

Certified that this thesis titled “Chemopreventive efficacy of Vitis vinifera gold nanoparticles in skin carcinogenesis: an in vitro and in vivo approach” is the bonafide work of J. GRACE NIRMALA who carried out the research under my supervision. Certified further, that to the best of my knowledge the work reported herein does not form part of any other thesis or dissertation on the basis of which a degree or award was conferred on an earlier occasion on this or any other scholar.

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ABSTRACT

Gold nanoparticles (AuNPs) are found to be beneficial for numerous promising applications in the field of biomedicine and in the development of therapeutic nanomedicine products. Several studies suggest that surface modifications by capping agents or surface coatings of gold nanoparticles can play an important role in biological systems and for site directed delivery. It is necessary and need of the hour to develop environmentally and biological friendly green processes for rapid synthesis of nanoparticles to avoid intervention of ‘man-made’ chemicals that will be coated onto the nanoparticles when chemicals are used as reducing agents. Plant mediated synthesis of gold nanoparticles is acquiring more significance due to ease and fast rate of synthesis along with cost effective and environmental friendly processes.

The phytochemicals contained within *Vitis vinifera* commonly known as grapes were utilised as reducing agents for the reduction of gold metal ions to the respective gold nanoparticles. Grapes are one of the most widely consumed fruits in the world and are rich in antioxidant abundant polyphenols. Thus, gold nanoparticles synthesized by utilizing *Vitis vinifera* phytochemicals can selectively target cancer cells and the phytochemicals that are occluded within the nanoparticles can serve as potential anticancer agents providing better efficacy in killing cancer cells. Oxidative stress can lead to DNA damage in the skin and uncontrolled release of reactive oxygen species can be the cause of skin cancer and it is involved in pathogenesis of number of human skin disorders.
The present study was carried out to assess the chemopreventive effects of *Vitis vinifera* peel and seed extracts and gold nanoparticles synthesized using *Vitis vinifera* seed and peel extracts on 7,12- dimethylbenz [a] anthracene (DMBA)-initiated and 12- O-tetradecanoylphorbol 13-acetate (TPA) tumor promoted model using Swiss albino mice and also in an *in vitro* model human epidermoid carcinoma A431 cell lines for antiproliferative effects and induction of apoptosis. The effects of *Vitis vinifera* seed and peel coated gold nanoparticles were tested for their immunomodulatory effects in Swiss albino mice.

The present study suggests that the aqueous extracts of *Vitis vinifera* seed and peel has potent *in vitro* antioxidant activities. High amounts of phenols, ascorbic acid and flavonoids present in seeds and peels may also be responsible for the *in vitro* antioxidant activity and thus they can be used as a free radical scavengers to protect against various damages initiated by free radicals. *Vitis vinifera* seed aqueous extract showed high amount of polyphenols and *in vitro* antioxidant activities when compared to *Vitis vinifera* peel extract.

The polyphenolic compounds exhibited a vital role as reducing, capping as well as stabilizing agents in the green synthesis process of gold nanoparticles. The synthesized gold nanoparticles were confirmed by UV-Visible Spectroscopy analysis with the conversion of yellow to deep purple red color and the particle size distribution showed particle size of ~ 50 ± 5 nm particles. Transmission electron microscopic (TEM) analysis showed the size and spherical shape of the gold nanoparticles. Fourier transform infrared spectroscopic analysis (FTIR) confirmed
the presence the polyphenols that were capped onto the peel and seed gold nanoparticles.

The gold nanoparticles synthesized biologically using *Vitis vinifera* peel and seed extracts were studied for their antiproliferative activities and induction of apoptosis. At the inhibitory concentration (IC₅₀), grape seed extract (111.11 µg/mL), grape seed AuNPs (24.2 µg/mL), grape peel extract (319.14 µg/mL) and grape peel AuNPs (23.6 µg/mL) were incubated for 24 hrs with A431 cells. *Vitis vinifera* peel and seed AuNPs were able to impart cytotoxic effects, induced apoptosis and apoptotic morphological changes in A431 cells significantly (*p*<0.01) and this effect is associated with the interference with mitochondrial membrane potential. This reduction in mitochondrial membrane potential probably initiated the apoptotic cascade in the nanoparticles treated cells.

Immunomodulatory activity of *Vitis vinifera* peel and seed AuNPs as well as peel and seed extracts showed that it may be due to the combined action of humoral and cell-mediated immune responses wherein the results indicated that the *Vitis vinifera* peel and seed AuNPs could act as a non-toxic immunomodulator by stimulating the production of white blood cells (WBC), hemoglobin content (Hb) and production of antibodies against Sheep red blood cells (SRBC) antigen significantly (*p*<0.05).

In the present study, DMBA as inducer (single application) and TPA (promoter) were applied on the dorsal area of the skin to induce skin cancer in Swiss albino mice for 16 weeks. On topical application, peel and seed gold nanoparticles demonstrated chemopreventive potential in DMBA induced skin
carcinogenesis by reducing the cumulative number of tumors in gold nanoparticles treated mice while increasing the antioxidant enzyme level in the treated groups significantly \((p<0.05)\). Histopathological evaluation showed that skin tissues from *Vitis vinifera* peel and seed AuNPs treated mice showed mild dysplasia and mild acanthosis. Severe hyperplasia, hyperkeratosis and well-differentiated squamous cell carcinoma were observed in all DMBA treated mice. Topical application of gold nanoparticles down regulated expression of mutant p53, Bcl-2 and pancytokeratin levels and may have facilitated the process of apoptosis in the chemical carcinogenesis process. The beneficial action of *Vitis vinifera* peel and seed AuNPs is probably due to its ability to stimulate the antioxidant enzymes in the cells and suppressed abnormal skin cell proliferation occurring during DMBA-induced skin papillomagenesis and demonstrated the chemopreventive potential of peel and seed gold nanoparticles in DMBA induced skin carcinogenesis than seed and peel aqueous extracts treated groups.

In conclusion, the present study suggests that *Vitis vinifera* seed and peel AuNPs possess chemopreventive potential against human epidermoid carcinoma cells (A431) *in vitro* and also against DMBA induced skin papillomagenesis with the mechanistic pathway of apoptosis both *in vitro* and *in vivo* studies. This can be due to the combined efficacy of gold nanoparticles in targeting the cancer cells and polyphenols that are capped on the nanoparticles providing chemopreventive effect on the cancer cells. This study will lead to developing a combination of drug using nanoparticles and polyphenols and thus enhancing the efficacy of a particular drug and its further use as a chemopreventive agent.
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7.7 Representative photomicrographs of immunohistochemical staining of immunoexpression of p53 in skin tissues of control and experimental mice (10X)

7.8 Representative photomicrographs of immunohistochemical staining of immunoexpression of BcL-2 in skin tissues of control and experimental mice (10X)

7.9 Representative photomicrographs of immunohistochemical staining of immunoexpression of pan cytokeratin in skin tissues of control and experimental mice (10X)
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Abbreviation</th>
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<tr>
<td>α</td>
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<td>β</td>
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<td>Percentage</td>
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<tr>
<td>≤</td>
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<tr>
<td>°</td>
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<tr>
<td>µg</td>
<td>Micro gram</td>
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<td>µL</td>
<td>Micro litre</td>
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<td>Annexin V-FITC-</td>
<td>Annexin V fluorescein isothiocyanate</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>AO</td>
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<td>APCs</td>
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<td>Aspartate aminotransferase</td>
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<td>Adenosine triphosphate</td>
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<td>BCC</td>
<td>Basal cell cancer</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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BSI - Botanical Survey of India
CAT - Catalase
CE - Catechin equivalent
cm - Centimeter
CO₂ - Carbon dioxide
DCF - 2′, 7′-di- chlorofluorescein
DCFH-DA - 2′, 7′- dichlorodihydro fluorescein diacetate
DLS - Dynamic light scattering
DMBA - 7, 12-dimethylbenz[a]anthracene
DMEM - Dulbecco's Modified Eagle Medium
DMSO - Dimethyl sulfoxide
DNA - Deoxyribonucleic acid
DNPH - 2, 4-dinitrophenyl hydrazine
DPPH - 2, 2-diphenyl-1-picrylhydrazyl
DTH - Delayed Type Hypersensitivity
EDX - Energy dispersive X-ray spectroscopy
EPR - Enhanced permeation and retention
ESR - Erythrocyte sedimentation rate
EtBr - Ethidium Bromide
FBS - Foetal Bovine Serum
FDA - Food and drug administration
FTIR - Fourier Transform Infrared Spectroscopy
g - Gram
GAE - Gallic acid equivalent
GC - Gas chromatography
GCMS - Gas Chromatography Mass Spectroscopy
GPx - Gluthathione peroxidase
GSH - Reduced glutathione
<table>
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<tr>
<th>Abbreviation</th>
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<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
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</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
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<td>SD</td>
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<td>SEM</td>
<td>Scanning Electron Microscope</td>
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