THYMOQUINONE AND ITS NANO-FORMULATIONS FOR DRUG DELIVERY

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by

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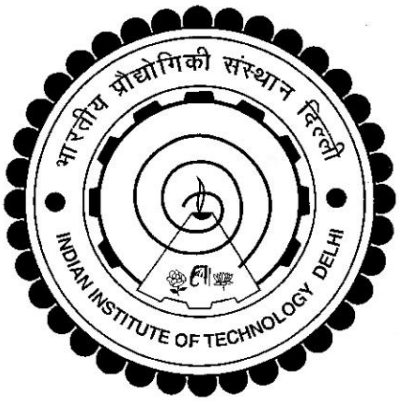
Submitted

in fulfilment of the requirements of the degree of Doctor of Philosophy

to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

JULY 2018

DEDICATED

TO

MY FATHER AND MY HUSBAND

CERTIFICATE

This is to certify that the thesis entitled "**Thymoquinone and its nano-formulations for drug delivery**" being submitted by **Ms. Surbhi Goel** is worthy of consideration for the award of the degree of **Doctor of Philosophy**. The thesis has been prepared by her under my supervision and guidance in conformity with the rules and regulations of Indian Institute of Technology Delhi and is a record of the original bonafide research work. The results presented in this thesis have not been submitted in part or full to any other universities or institutes for the award of any other degree or diploma.

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"Showing gratitude is one of the simplest yet most powerful things humans can do for each other."

-Randy Pausch, The Last Lecture

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ABSTRACT

Now-a-days plant-based phytochemicals are gaining much importance over conventional therapeutics owing to their multifunctionality and low toxicity. Thymoquinone (TQ), is one such phytochemicals which possess remarkable anti-cancer activity and known for its anti-inflammatory, anti-oxidant, anti-fungal and anti-bacterial activity. It is isolated from the volatile oil fraction of *Nigella sativa.* In the present study, antibacterial, antibiofilm activity of TQ and mechanism of action has been explored. Antibacterial activity of TQ was studied by determining minimum inhibitory concentration, minimum bactericidal concentration, time-kill assay and post-antibiotic effect against two Gram-negative and two Gram-positive bacteria. The minimum inhibitory concentration of TQ was found to be in the range of 1.56 µg/ml to 100 µg/ml. TQ treated bacterial cells at MIC were visualized using scanning electron microscopy which revealed changes in cell morphology, cell lysis and cell size reduction. Live/dead imaging confirmed the bactericidal activity of TQ as treated bacteria showed uptake of ethidium bromide over acridine orange. Selectivity of TQ towards bacterial cell was observed by studying its toxicity towards HaCaT (human keratinocytes) cell line by MTT assay. IC_{90} value was found to be 50µg/ml which was higher than that of $MIC_{bacteria}$ (except for MIC of *E. coli*). TQ also showed promising anti-biofilm activity against both Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive bacteria (*B. subtilis* and *S. aureus*), which was studied by crystal violet assay, MTT assay, CFU counting and SEM. TQ also exhibited anti-biofilm activity against preformed biofilm i.e. 6 h old and 24 h old. For understanding the antibacterial mechanism of action of TQ, $DiSC_3$, NPN and ROS assays were performed. $DiSC_3$ and NPN assays have not shown any membrane damage caused by TQ. However, bacterial cells treated with TQ at MIC showed increased dichlorofluorescin fluorescence suggesting the production of reactive oxygen species. This was further confirmed by incubating bacteria at

MIC of TQ in the presence of reduced glutathione, a known ROS scavenger. Glutathione caused attenuation of TQ's antibacterial activity confirming that ROS generation could be the probable mechanism for TQ antibacterial action. In addition to TQ's antibacterial activity its potential application as anticancer agent has also been studied. In this study, nanoencapsulation of TQ in PLGA and mesoporous silica nanoparticle was carried out, to overcome the limitations of using TQ in its free form. PLGA was employed as a nanocarrier because of its biocompatibility, biodegradability and property of sustained release of drug over a long period of time. Iron-oxide nanoparticles of size 6-10 nm has also been incorporated in PLGA along with TQ to obtain the benefit of targeted drug delivery under the influence of external magnetic field and also modulate of drug release kinetics employing hyperthermia. On the other hand, advantages with mesoporous silica nanoparticles are non-toxicity, high surface area to volume ratio, high porosity and chemical stability which leads to high loading efficiency of hydrophobic drugs. Successful encapsulation of TQ within PLGA and mesoporous silica nanoparticles has been achieved with size in the range of 100 nm- 300 nm. Both TQ nano-formulations showed cytotoxicity against HeLa and MCF-7 cell lines at lower concentration of TQ as compared to the free TQ. Anticancer activity of TQ loaded PLGA and MSNPs were studied by DAPI staining and Annexin V-FITC/PI staining and it was found that both nano-formulations exhibit anticancer activity by inducing apoptosis. While PLGA appears to be ideal formulation for long term delivery of TQ, mesoporous silica nanoparticles have shown advantages of high loading efficiency, low cost and short-term delivery of hydrophobic drugs.

सार

आजकल पौधों से प्राप्त किये गए फाइटोकेमिकल्स अपने बहुआयामी और कम विषाक्तता के कारण परंपरागत चिकित्सा पर अधिक महत्व प्राप्त कर रहे हैं। थाइमोक्विनोन, एक ऐसा फाइटोकेमिकल् है जिसमें उल्लेखनीय एंटी-कैंसर गतिविधि होती है और इसको एंटीइंफ्लामेटरी, एंटीऑक्सीडेंट, एंटीफंगल और एंटीबैक्टीरियल गतिविधि के लिए जाना जाता है। यह निगेला सटाइवा के वाष्पशील तेल अंश से अलग किया है। वर्तमान अध्ययन में, थाइमोक्विनोन की एंटीबैक्टीरियल, एन्टीबायोफिल्म गतिविधि और क्रिया के तंत्र की खोज की गई है। थाइमोक्विनोन की जीवाणुरोधी गतिविधि का अध्ययन, दो ग्राम-निगेटिव और दो ग्राम-पॉजिटिव बैक्टीरिया के खिलाफ, न्यूनतम अवरोधक सांद्रता , न्यूनतम जीवाणुनाशक सांद्रता , टाइम किल परख का अनुमान लगा के किया गया है, जिसमे थाइमोक्विनोन की न्यूनतम अवरोधक सांद्रता 1.56 µg/ml से 100 µg/ml तक पायी गई । बैक्टीरियल कोशिकाओं में थाइमोक्विनोन के न्यूनतम अवरोधक सांद्रता से ट्रीटमेंट के बाद के असर का अध्यन करने के लिए, स्कैनिंग इलेक्ट्रॉन माइक्रोस्कोपी का उपयोग करके देखा गया , जिसमें कोशिका अपघटन, कोशिकाओं की आकृति और आकार में बदलाव पाया गया । जीवित / मृत इमेजिंग से भी थाइमोक्विनोन की जीवाणुनाशक गतिविधि की पुष्टि की गयी, जिसमे थाइमोक्विनोन से ट्रीटमेंट के बाद बैक्टीरियल कोशिकाओं ने एक्रिडीन ऑरेंज पर इथिडियम ब्रोमाइड का अंतर्ग्रहण दिखाया। सेलेक्टिव टॉक्सिसिटी की परख के लिए HaCaT (मानव केराटिनोसाइट्स) कोशिकाओं की ओर, थाइमोक्विनोन की विषाक्तता का अध्ययन एम. टी. टी. परख द्वारा करके देखा गया, इस अध्यन में IC_{90} सांद्रता 50 µg/ml पाया गया जो कि न्यूनतम अवरोधक सांद्रता बैक्टीरिया से अधिक हैं (ई. कोलाई के न्यूनतम अवरोधक सांद्रता को छोड़कर)। थाइमोक्विनोन ने ग्राम-निगेटिव और ग्राम-पॉजिटिव दोनों ही बैक्टीरिया के खिलाफ एंटीबायोफिल्म गतिविधि को भी दिखाया, जिसका अध्ययन क्रिस्टल वायलेट परख, एम. टी. टी. परख, कॉलोनी फॉर्मिंग यूनिट गिनती और स्कैनिंग इलेक्ट्रॉन माइक्रोस्कोपी द्वारा किया गया । थाइमोक्विनोन ने सुनिर्मित बायोफिल्म, यानी 6 घंटे पुरानी और 24 घंटे पुरानी के खिलाफ भी एंटी-बायोफिल्म गतिविधि दर्शायी । थाइमोक्विनोन के जीवाणुरोधी तंत्र को समझने के लिए, $DISC_3$, N-फिनाइल-1-नाफ्थिलाअमिन और प्रतिक्रियाशील ऑक्सीजन प्रजातियां परख द्वारा अध्ययन किया गया। थाइमोक्विनोन के

ट्रीटमेंट के पश्चात्, $DISC_3$ और N-फिनाइल-1-नाफ्थिलाअमिन परख द्वारा कोई मेम्ब्रेन क्षति नहीं प्राप्त हुई । हालांकि, न्यूनतम अवरोधक सांद्रता में थाइमोक्विनोन के साथ ट्रीटमेंट किए गए जीवाणु कोशिकाओं में डी.सी.एफ.-डी.ए. परख द्वारा प्रतिक्रियाशील ऑक्सीजन प्रजातियों के उत्पादन में वृद्धि देखी गया । ग्लूटाथिओन की उपस्थिति में थाइमोक्विनोन की एंटीबैक्टीरियल गतिविधि की प्रतिबंधन की पुष्टि हुई और इस अध्यन से ये पुष्टि होती हैं कि थाइमोक्विनोन द्वारा प्रतिक्रियाशील ऑक्सीजन प्रजातियों का उत्पादन, एंटीबैक्टीरियल क्रिया के लिए संभावित तंत्र हो सकता है। थाइमोक्विनोन की जीवाणुरोधी गतिविधि के अलावा एंटीकेंसर एजेंट के रूप में इसके संभावित अनुप्रयोग का भी अध्ययन किया गया है। इस अध्ययन में, थाइमोक्विनोन का नैनोएंकैप्सूलेशन, पी.एल.जी.ए और मेसोपोरस सिलिका नैनोकण में किया गया, जो की थाइमोक्विनोन के मुक्त रूप में उपयोग करने की सीमाओं को दूर करने के लिए सहायक साबित होगी। पी.एल.जी.ए को निम्न कारणों की वजह से नैनोकैरियर के रूप में नियोजित किया गया: लंबे समय तक दवा का निरंतर रिलीज, बायोकम्पेटिबिलिटी और बायोडिग्रेडेबिलिटी । बाहरी चुंबकीय क्षेत्र के प्रभाव में लक्षित दवा वितरण के लाभ प्राप्त करने के लिए थाइमोक्विनोन के साथ पी.एल.जी.ए में 6-10 nm आकार के आयरन-ऑक्साइड नैनोकणों को भी शामिल किया गया है और हाइपरथेरमिआ द्वारा दवा के रिलीज काइनेटिक्स का भी संशोधन किया गया है। दूसरी तरफ, मेसोपोरस सिलिका नैनोकणों के साथ, गैर-विषाक्तता, उच्च सतह क्षेत्र / मात्रा अनुपात, उच्च संरध्रता और रासायनिक स्थिरता जैसे फायदे है जो हाइड्रोफोबिक दवाओं की उच्च लोडिंग दक्षता के लिए उपयोगी हैं । पी.एल.जी.ए और मेसोपोरस सिलिका नैनोकणों के भीतर थाइमोक्विनोन के सफल समावेशन से 100 nm -300 nm की सीमा में आकार के नैनोकण प्राप्त किये गए । मुक्त थाइमोक्विनोन की तुलना में दोनों ही थाइमोक्विनोन नैनो-फॉर्मूलेशन ने थाइमोक्विनोन की कम सांद्रता पर HeLa और MCF-7 कोशिकाओं के खिलाफ साइटों टोक्सीसिटी दिखायी। थाइमोक्विनोन लोड किए गए पी.एल.जी.ए और मेसोपोरस सिलिका नैनोकणों की एंटीकैंसर गतिविधि का अध्ययन डी.ए.पी.आई. स्टेनिंग और अन्नेक्सीन V- एफ.आई.टी.सी. और प्रोपीडियम आयोडाइड स्टेनिंग द्वारा किया गया और यह पाया गया कि नैनो-फॉर्मूलेशन एपोप्टोसिस को प्रेरित करके एंटीकेंसर गतिविधि दर्शाते हैं । पी.एल.जी.ए. थाइमोक्विनोन की लंबी अवधि के वितरण के लिए आदर्श फॉर्मूलेशन प्रतीत होता है, मेसोपोरस सिलिका नैनोकणों ने हाइड्रोफोबिक दवाओं की उच्च लोडिंग दक्षता, कम लागत और अल्पकालिक वितरण के फायदे दिखाए हैं।

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AGM	Alternate gradient magnetometer
AO	Acridine orange
ATCC	American Type culture collection
BIC	Biofilm inhibitory concentration
BMSNPS	Blank mesoporous silica nanoparticles
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
Co	Coumarin-6
Co-MSNPs	Coumarin-6 loaded mesoporous silica nanoparticles
CTAB	Cetyltrimethylammonium bromide
DAPI	(4',6-diamidino-2-phenylindole)
DCF	2,7-dichlorofluorescein
DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
DCFH	2,7-dichlorodihydrofluorescein
DCM	Dichloromethane
$DiSC_3(5)$	3,3′- dipropylthiacarbocyanine
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EtBr	Ethidium bromide
EDTA	Ethylene diamine tetra acetic acid
FBS	Fetal bovine serum
FDA	Food and Drug Administration

FESEM	Field emission scanning electron microscope
FI	Fluorescence intensity
FITC	Fluorescein isothiocyanate
FTIR	Fourier-transform infrared spectroscopy
g	Relative centrifugal force
GSH	Reduced Glutathione
h	Hour
HaCaT	Immortalized human epidermal keratinocytes
HeLa	Human epithelial cervical adenocarcinoma
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC_{50}	Concentration at which 50% inhibition takes place
IC_{90}	Concentration at which 90% inhibition takes place
IONP	Iron-oxide nanoparticles
IONP-PLGA	Iron oxide nanoparticle loaded PLGA nanoparticles
LB	Luria broth
m	Milli
M	Molar
mM	Millimolar
ml	Milliliter
mg	Milligram
MBC	Minimum bactericidal concentration
MBEC	Mature biofilm eradication concentration
MCF-7	Human epithelial breast adenocarcinoma
MCM 41	Mobil composition of matter No. 41
MHA	Mueller hinton agar
MHB	Mueller hinton broth

MIC	Minimum inhibitory concentration
Ms	Saturation magnetization
MSNPs	Mesoporous silica nanoparticles
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nm	Nanometer
NP	Nanoparticle
NPN	1-N-phenylnaphthylamine
NPs	Nanoparticles
OD	Optical density
OA	Oleic acid
OA-IONP	Oleic acid coated iron oxide nanoparticles
OH^-	Hydroxyl radical
PAE	Post antibiotic effect
PBS	Phosphate buffer saline
PDI	Polydispersity index
PI	Propidium iodide
PLGA	Poly (D, L-lactide-co-glycolic) acid
PS	Phosphatidylserine
PVA	Poly vinyl alcohol
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
s	seconds

SD	Standard deviation
SEM	Scanning electron microscope
SPION	Superparamagnetic iron oxide nanoparticles
TEM	Transmission electron microscope
TEOS	Tetraethyl orthosilicate
TQ	Thymoquinone
WHO	World Health Organization
w/v	weight per unit volume
v/v	Volume per unit volume
µ	Micro
µl	Microliter
µg	Microgram
°	Degree Celsius
%	percent
λ	Wavelength