FORMULATION AND EVALUATION OF NANOEMULGEL (IN SITU) NIGELLA SATIVA-LOADED CARBON DOTS FOR POTENTIAL PERIODONTAL TREATMENT: AN IN VITRO STUDY

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by

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LIST OF ABBREVIATIONS

ABTS	Azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
AE	Apple Extract
AFM	Atomic Force Microscopy
ASE	Accelerated Solvent Extraction
BSA	Bovine Serum Albumin
CD	Carbon Dots
CFU	Colony Forming Units
CHX	Chlorhexidine gluconate
CP	Cold Press
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle Medium
DOE	Design Of Experiments
DPPH	Diphenyl-1-picrylhydrazyl
DMSO	Dimethoxy sulfoxide
EDX	Energy Dispersive X-ray Spectroscopy
FACDs	Fluorescent Aspirin-based Carbon Dots
FBS	Fetal Bovine Serum
FRAC	Ferric Ion Reducing Antioxidant Capacity
F-C	Folin–Ciocalteu
FTIR	Fourier Transformer Infrared
GAE	Gallic Acid Equivalent
GCF	Gingival Crevicular Fluid
GC-MS	Gas Chromatography-Mass Spectrometry
GQDs	Graphene Quantum Dots

HGFs	Human Gingival Fibroblasts
HLB	Hydrophilic-Lipophilic Balance
HRBC	Human Red Blood Cell
HRTEM	High-Resolution Transmission Electron Microscopy
ISO	International Organization for Standardization
MAE	Microwave-Assisted Extraction
MBC	Minimum Bactericidal Concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MIC	Minimum Inhibitory Concentration
Ml	Milliliter
Mm	Millimolar
Mg	Milligram
MV	Millivolts
NADH	Nicotinamide Adenine Dinucleotide
Nes	Nanoemulsions
NEGs	Nanoemulgels
NEG-IS	Nano-emulgel in-situ
NBT	Nitro-Blue Tetrazolium
NIST	National Institute of Standards and Technology
NPs	Nanoparticles
NSEO	N. sativa Essential Oil
OD	Optical Density
PBS	Phosphate Buffered Saline
PDs	Polymer Dots
PMS	Phenazine Methosulphate

PL	Photoluminescence
QbD	Quality by Design
QE	Quercetin Equivalents
ROS	Reactive Oxygen Species
RSD	Relative Standard Deviation
SAED	Selected Area Electron Diffraction
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SFE	Supercritical Fluid Extraction
SLS	Sodium Lauryl Sulphate
SOD	Superoxide Dismutase
TEM	Transmission Electron Microscopy
TFC	Total Flavonoid Contents
TPC	Total Phenol Content
TQ	Thymoquinone
TSC	Total Steroid Contents
TTC	Total Terpenoids Contents
TCA	Trichloro Acetic Acid
UV-Vis	UV-Visible Spectroscopy
WHO	World Health Organisation
XRD	X-ray Diffraction
ZI	Zone of Inhibitions
ZP	Zeta Potential

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FORMULASI DAN PENILAIAN NANOEMULGEL (IN SITU) BERMUATAN TITIK KARBON NIGELLA SATIVA UNTUK POTENSI RAWATAN PERIODONTAL: KAJIAN IN VITRO

ABSTRAK

Penyakit periodontal adalah keadaan radang pada gingiva dan/atau periodontium yang mengakibatkan pemusnahan aparatus perlekatan dan seterusnya kehilangan gigi. Kajian ini bertujuan untuk merumuskan dan menilai sifat bioperubatan sistem penghantaran ubat nanoemulgel *in-situ* (NEG-IS) yang baru dengan menggabungkan minyak pati *Nigella sativa* (NSEO) dan titik karbon (CDs). Minyak pati diekstrak daripada biji *Nigella sativa* (*N. sativa*) melalui kaedah hidro-distilasi. CDs dihasilkan secara hijau daripada halia menggunakan kaedah hidroterma. Untuk mendapatkan kepekatan optimum NSEO dan CDs bagi formulasi nanoemulgel, pengoptimuman dilakukan menggunakan tiga nisbah NSEO yang berbeza; 9:1, 1:1, 1:9, dan dinilai untuk sifat antioksidan serta anti-radang. Sistem gel NEG-IS dirumuskan melalui pengoptimuman menggunakan reka bentuk faktorial penuh dua faktor (poloxamer dan carbopol) dengan tiga tahap (rendah, sederhana dan tinggi), yang merangkumi reka bentuk statistik dengan sembilan ulangan. Analisis kandungan fitokimia untuk NSEO dijalankan menggunakan Kromatografi Gas Spektrometri Jisim Bertindan (GC-MS/MS) diikuti dengan pencirian analitik menggunakan UV-Vis Spektrometri, FT-IR Spektrofotoskopi, dan pengukuran Potensi Zeta (ZP). Pencirian CDs merangkumi penilaian saiz zarah, profil pendarfluor, Mikroskop Elektron Transmisi Resolusi Tinggi Mikroskop Elektron Imbasan - Spektroskopi Penyebaran Tenaga Sinar-X, Mikroskopi Daya Atom, Pembelauan Sinar-X, Spektrofotometri UV-Vis, Spektrofotoskopi FT-IR, dan

pengukuran ZP. Pencirian fizikokimia NEG-IS dilakukan untuk menilai pH, kelikatan, masa pengegelan, suhu pengegelan, kebolehan merebak, kebolehan suntik, dan kapasiti pengegelan *in-vitro*. Pencirian lain yang dilakukan termasuk pengedaran saiz globul, kinetik pelepasan ubat *in-vitro*, spektroskopi UV-Vis, FTIR, dan pengukuran ZP. Kajian kestabilan lanjut untuk NEG-IS dijalankan selama 1.5 bulan. Sifat antioksidan, anti-radang, dan antimikrob terhadap patogen oral, kulat, dan periodontal terpilih dinilai untuk NSEO, CDs, dan NEG-IS. Sitotoksisiti NSEO, CDs, dan NEG-IS dinilai ke atas titisan sel fibroblas gingiva manusia (HGF). Kehadiran 17 sebatian bioaktif dalam NSEO disahkan oleh GC-MS/MS. CDs menunjukkan saiz purata zarah sebesar 2.9 nm dan hasil pencirian lain mengesahkan kehadiran CDs. NEG-IS menunjukkan ciri fizikokimia yang mencukupi dan pelepasan berterusan ubat sehingga 12 jam, mengikuti kinetik model Korsmeyer-Peppas (K-P) yang selari dengan kecenderungan Fickian. NEG-IS adalah stabil pada suhu tinggi dan suhu bilik tanpa perubahan ketara dalam penampilan gel. NSEO, CDs, dan NEG-IS menunjukkan sifat bioperubatan yang signifikan dan mempunyai kesan sitotoksik yang rendah terhadap titisan sel HGF. Formulasi NEG-IS yang dibangunkan menunjukkan sifat bioperubatan *in-vitro* dengan potensi untuk digunakan sebagai formulasi terapeutik dalam kajian klinikal pada masa hadapan.

FORMULATION AND EVALUATION OF NANOEMULGEL (IN SITU) WITH NIGELLA SATIVA - LOADED CARBON DOTS FOR POTENTIAL PERIODONTAL TREATMENT: AN IN VITRO STUDY

ABSTRACT

Periodontal disease is an inflammatory condition of the gingiva and/or periodontium resulting in destruction of attachment apparatus and subsequently tooth loss. This study aimed to formulate and evaluate the biological properties of a novel nanoemulgel *in-situ* (NEG-IS) drug delivery system incorporating *Nigella sativa* essential oil (NSEO) and carbon dots (CDs). Essential oil was extracted from *Nigella sativa* (*N. sativa*) seeds by hydro-distillation method. CDs were green synthesized from ginger by hydrothermal method. To obtain optimum concentration of NSEO and CDs for nanoemulgel formulation, optimization was performed using three different NSEO:CDs ratios; 9:1, 1:1, 1:9 and assessed for antioxidant and anti-inflammatory properties. NEG-IS gelling system was formulated by optimization using a three level (low, medium and high) with two factors (poloxamer and carbopol) full factorial design, that included a statistical design with nine runs. The phytochemical constituents' analysis for NSEO was performed using Gas Chromatography Tandem Mass Spectrophotometry (GC-MS/MS) followed by the analytical characterization using UV-Vis Spectrophotometry, FT-IR Spectroscopy and Zeta Potential (ZP) measurement. CDs characterization included particle size evaluation, fluorescence profiling, HR-TEM, SEM-EDX, AFM, XRD, UV-Vis Spectrophotometry, FT-IR Spectroscopy and ZP measurement. Physico-chemical characterization of NEG-IS was done to assess its pH, viscosity, gelation time, gelation temperature, spreadability, syringeability and *in-vitro* gelling capacity. Other characterizations included globule size distribution, *in-vitro* drug release kinetics, UV-Vis spectroscopy FTIR and ZP measurement. Further stability studies for NEG-IS were performed for a period of 1.5 months. Antioxidant, anti-inflammatory and antimicrobial properties against selected oral, fungal and periodontal pathogens were assessed for NSEO, CDs and NEG-IS. Cytotoxicity of NSEO, CDs and NEG-IS was assessed over human gingival fibroblast (HGF) cell lines. Presence of 17 bioactive compounds in NSEO was confirmed by GC-MS/MS. CDs exhibited mean particle size of 2.9 nm and other characterization results confirm presence of CDs. NEG-IS exhibited adequate physico-chemical characteristics and sustained release of the drug for up to 12 hours, which followed the Korsmeyer-Peppas (K-P) model kinetics that aligns with Fickian tendencies. NEG-IS was stable at elevated temperature and room temperature with no significant changes in gel appearance. NSEO, CDs and NEG-IS exhibited significant biological properties and had less cytotoxicity effects over HGF cell lines. The developed NEG-IS formulation showed biological properties *in-vitro* with the potential to be utilized as therapeutic formulation in future clinical studies.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Periodontal disease is an inclusive term for the inflammatory condition of gingiva (gingivitis) and/or periodontium (periodontitis). The disease process progresses from gingivitis to periodontitis. In its early stage, called gingivitis, the gingiva becomes swollen, red and may bleed. This gingival inflammation can subsequently extend to involve the periodontal attachment apparatus and lead to alveolar bone destruction (Pizzorno *et al.,*2016). Not only have periodontal diseases impacted 20–50% of the population, but they are also the leading cause of tooth loss in both developed and developing countries (Nazir, 2017).

The destructive forms of periodontal disease known as periodontitis, are characterized by inflammation and the loss of the periodontal attachment apparatus in adolescents and adults. The cardinal signs of periodontitis include attachment loss, alveolar bone loss, periodontal pocketing, and gingival inflammation. In the context of the 2017 World Workshop Classification for periodontal and peri-implant diseases and conditions, a staging and grading system has been provided as a part of the case definition system. Based on the severity and complexity of management, it has been staged into four stages, where Stage I includes initial periodontitis, Stage II involves moderate periodontitis, Stage III includes severe periodontitis with potential for additional tooth loss and Stage IV includes advanced tooth loss with potential for loss of dentition (Tonetti *et al.,*2018).

In most cases, periodontal attachment loss can be arrested or prevented by mechanical and surgical treatment that lowers the overall supra-subgingival bacterial mass in conjunction with good oral hygiene practices (Slots, 2004; Winkelhoff *et al.,*1996). Over the past two decades, periodontal antibiotic therapy such as amoxicillin and metronidazole (500 mg thrice daily for 7 days) has gained popularity among dentists and microbiologists as a powerful adjunct to the conventional mechanical debridement in the therapeutic management of periodontal diseases (Slots and Ting, 2002).

Despite thorough dental therapy, certain individuals may continue to experience periodontal breakdown. This persistent destruction may be attributed to pathogenic microorganisms invading the periodontal tissues, colonizing inaccessible areas such as periodontal pockets, furcations, or other complex tooth structures beyond the reach of conventional periodontal instruments, or due to compromised host immune responses (Hammami and Nasri, 2021). Periodontal disease involves five main bacterial complexes with varying pathogenicity. The “red complex” includes highly pathogenic bacteria *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia* (*T. forsythia*) and *Treponema denticola* (*T. denticola*) which are linked to severe periodontal disease. The “orange complex” includes *Fusobacterium nucleatum* (*F. nucleatum*) and *Prevotella intermedia* (*P. intermedia*) bridges early colonizers and more harmful bacteria. The “green complex” (*Capnocytophaga* species, *Eikenella corrodens*) consists of early colonizers that are less associated with severe disease, while the “yellow complex” (*Streptococcus* species) includes less virulent bacteria commonly found in early plaque. The “purple complex” (*Veillonella parvula, Actinomyces odontolyticus*) contains bacteria typically found in health or early disease stages (Socransky *et al.,*1998). Increased levels of the “red complex” organisms have been detected in stage III and IV periodontitis cases. *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), on the other hand, is frequently related to molar-incisor stage IV periodontitis and selected cases of stage III and IV periodontitis (Saquib *et al.,*2019). Furthermore, *P. intermedia* is found to be associated with stage III periodontitis (Zhao *et al.,*2022).

The rationale for periodontal therapy is to create a “biologically acceptable” root surface by eliminating etiological bacterial by-products (Spellberg *et al.,*2013). Mechanical debridement (scaling and root planing) is the mainstay for the prevention and treatment of periodontal disease; in addition to that, chemical plaque control measures can act as adjuvants for maintaining long-term results. Plaque control agents are crucial for maintaining oral hygiene and preventing dental diseases. These agents work by inhibiting bacterial growth, reducing bacterial adhesion to tooth surfaces, and maintaining their activity over time (substantivity). They possess antimicrobial properties that target plaque-forming bacteria. Effective plaque control agents are also designed for ease of use, ensuring they can be easily incorporated into daily oral care routines, maximizing their preventive benefits (Jafer *et al.,*2016). The most common and extensively studied chemical plaque control agent is chlorhexidine gluconate.

Chlorhexidine gluconate is considered as the gold standard plaque control agent because it has most of the features of the ideal antimicrobial solution, such as a broad spectrum of action and substantivity. Studies have revealed that long-term use of chlorhexidine mouthwash is associated with several adverse effects, like staining of the teeth, loss of taste sensation, degeneration of tongue papilla, and in rare cases parotid swelling (McCoy *et al.,*2008, Bhavikatti, *et al.,*2021). Due to the problems related to the indiscriminate use of antimicrobials (especially systemic side effects, microbiological adverse effects, and the increase in bacterial resistances), the use of systemic antimicrobial agents in periodontitis should be restricted to certain patients and certain periodontal conditions (e.g., aggressive periodontitis, severe and progressing forms of periodontitis) (Barça *et al.,*2015).

Herbal plants such as *Salvadora persica* (Miswak) and *Cinnamomum zeylanicum* (Ceylon cinnamon), as reviewed by Saquib et al., (2019), have been traditionally used for therapeutic purposes since ancient times. They are recognized for being safe, cost-effective, efficacious, and readily accessible. *Nigella sativa* (*N. sativa)*, of the Ranunculaceae family, is a herb with a rich historical background. The seeds and oil of *N. sativa* are used as antihypertensives, liver tonics, diuretics, antidiarrheal, analgesic, antibacterial, and antifungal agents (Ahmad *et al.,*2013). *Nigella sativa* oil contains thymoquinone, which shows positive effects in periodontal therapy using a 0.2% gel (2 mg/g) of 99% pure extract (Kapil *et al.,*2018). *N. sativa* has been shown to have pharmacological properties (antibacterial and anti-inflammatory) that makes it a viable dental therapeutic agent for the treatment of periodontal diseases (Sultan *et al.,*2022, Bordoni*et al.,*2019). Further, its non-toxic effects over human gingival fibroblasts (HGFs) makes it a potential adjuvant in periodontitis management (Setiawatie *et al.,*2022).

Carbon dots (CDs) are an emerging class of nanomaterials and have generated much interest in the field of biomedicine by way of unique properties, such as superior biocompatibility, stability, excellent photoluminescence, and simple green synthesis (Khan *et al.,* 2021). CDs derived from herbal sources such as ginger, lemon, onion etc., have revealed potent anti-inflammatory, antioxidant, and antimicrobial properties in previous studies (Ghirardello *et al.,*2021; Rodríguez-Varillas *et al.,*2022; Sharma *et al.,*2023b). Ginger (*Zingiber officinale*) is widely recognized for its antioxidative, anti-inflammatory and antibacterial properties, primarily due to its bioactive compounds, such as gingerols and shogaols, zingerone, and curcumene. These compounds contribute to ginger's therapeutic effects by modulating the body's inflammatory responses and scavenging free radicals, which helps reduce oxidative stress (Ayustaningwarno *et al.,*2024, Zhang *et al.,*2023). Further CDs have been synthesized from ginger using a hydrothermal process, where dried and powdered ginger was mixed with water and heated at 180°C in an autoclave followed by filtration and purification to obtain fluorescent CDs (Li *et al.,*2014). However, there is no report of green-synthesized CDs with biological properties in the management of oral or periodontal disease.

Nanoemulgels can be considered as innovative drug delivery systems in the development of topical formulations for pharmaceutical and dental applications. An oil-in-water or water-in-oil emulsion integrated into a gel base is known as a nanoemulgel. For the applicability of an adequate nanoemulgel in periodontal disease as a local drug delivery agent, a desirable intra-pocket delivery system should be a low viscosity fluid for better penetration at the sites of infection. Additionally, to be retained in the periodontal pockets, the system should possess good adhesiveness (Swain *et al.,*2019).

A plethora of local drug delivery systems are available in the management of periodontal disease including fibres, stripes, films, and microparticulate systems. However, they are associated with major disadvantages such as patient discomfort, gingival redness, and poor retention in periodontal pockets (Panwar and Gupta 2009; Pichayakorn and Boonme 2013; He *et al.,*2022; Soni *et al.,*2022). *In-situ* forming gels are a promising mode of local drug delivery systems that are in the form of sol before administration and transform into a gel or solid depot gradually after application. Therefore, they have the potential to maintain high levels of the drug in the gingival crevicular fluid for extended periods of time (Juvekar and Kathpalia, 2017). It is noteworthy that only a handful of studies have assessed the potential applicability of carbon nanodots in biological properties related to oral and periodontal diseases. To the best of our knowledge, no *in-vitro* studies in dentistry have reported the use of *N. sativa* essential oil loaded with carbon nanodots incorporated as an *in-situ* gelling system for the evaluation of its biological properties.

1.2 Problem statement

Constant inflammation of the gingiva can eventually cause periodontal pockets, which are then filled with more bacteria and plaque. Without treatment, one is at high risk of tooth and tissue loss. Redness, swollen, or tender gingiva that bleeds easily, painful chewing, tooth mobility, receding gingiva, sensitive teeth, and bad breath are the major signs and symptoms of periodontitis. Several procedures have been used to treat periodontitis, such as scaling and root planing, the use of bone grafts, and guided tissue regeneration (Cho *et al.,*2021). Some patients may not respond adequately, due to the impossibility of accessing areas such as deep pockets, furcation or root depression, and the limited effect over specific pathogens invading soft tissues (Jain *et al.,*2008). Adjunctive use of chemotherapeutic agents, such as systemic or local antibiotics or topical antiseptics has been indicated to improve the clinical outcomes in certain patients and periodontal conditions to avoid the need of surgical therapy (Graziani *et al.,*2017). The benefits of systemic antimicrobials have been reported in several systematic reviews and meta-analysis (Keestra *et al.,*2015); nevertheless, it can lead to side effects and development of resistance or overgrowth of other pathogens (Maestre *et al.,*2007). Local application of antibacterial agents into periodontal pockets involves drug delivery systems (irrigating systems, gels, fibers, strips/films, inserts, microparticles, and so forth), which would ideally allow an easy reach of the targeted site, a retentivity at an effective concentration and a controlled release for an adequate period (da Rocha Júnior *et al.,*2015). However, some issues still exist such as difficult placement, uncontrolled drug release, rapid local drug clearance and need to remove non-biodegradable form (Qin *et al.,*2012).

In recent years, there has been growing interest in nanotechnologies in medicine and dentistry. Nanoparticles (NPs) have a unique combination of properties that make them suitable for various dental applications (Mercado *et al.,*2019). A proper retention of the drug and the ease of administration, followed by minimum or no adverse effects is the prerequisite for a suitable local drug delivery system. Therefore, the development of a safe plant-based drug delivery system utilizing the benefits of nanoscience in an appropriate form could resolve the problems associated with the conventional drug delivery systems that are already available.

1.3 Justification of the study

This study aims to explore the novel *in-situ* gelling system incorporating the safe herbal essential oil derived from *N. sativa* seeds loaded with CDs as a possible and promising local drug delivery system in the management of periodontal disease. Owing to the beneficial properties of *N. sativa* as a herbal alternative, it was used as the herb in the formulation of *in-situ* gel in our study.

Further CDs synthesized from ginger were used as NPs as there is no reported study in dentistry regarding the same. Some recent studies have explored the potential of CDs in periodontitis. In one interesting study, metformin carbon dots (MCDs) with excellent biocompatibility are prepared from metformin hydrochloride and citric acid via a hydrothermal method.

The MCDs effectively enhanced the alkaline phosphatase activity, calcium deposition nodules formation, and expression of osteogenic genes and proteins in rat bone marrow mesenchymal stem cells than metformin under both inflammatory and normal conditions (Ren *et al.,*2021). Further fluorescent aspirin-based carbon dots (FACDs) synthesized by microwave-assisted method also exhibited effective anti-inflammatory effects *in-vitro* and *in-vivo* compared to aspirin only.

FACDs also had no significant toxicity *in-vivo* (Xu *et al.,*2016). Notably, carbon-based nanomaterials have shown promising results and hence can be explored further in periodontal diseases. The role of CDs in local drug delivery systems comprising plant-based extracts in the treatment of periodontitis along with its cytocompatibility assessment over oral cells (gingival fibroblasts) remains unexplored. For this reason, this study may provide outcomes that can assist the periodontist to manage periodontitis. The study focuses on the development and evaluation of a novel nanoemulgel *in-situ* gelling system with *N. sativa* essential oil (NSEO) and NPs in the form of CDs. This research will pave a direction towards a unique and potential local drug delivery system in the management of periodontal disease.

1.4 Research Questions

1. What are the phytochemical compounds of NSEO and do they have the biological properties including antioxidant, anti-inflammatory and antimicrobial properties relating to oral or periodontal disease?
2. Is the extracted NSEO cytotoxic on the HGF cell lines?
3. Is it possible to synthesize CDs from ginger by the green synthesis method.
4. What are the physicochemical and biological properties of CDs?

5. Are CDs cytotoxic on the HGF cell lines?

6. What is the optimum concentration of gelling agents, Carbopol, and Poloxamer in the formulation and optimization of a novel nanoemulgel *in- situ* (NEG-IS) containing the optimized concentration of NSEO loaded with ginger-based CDs?

7. What are the physicochemical, antioxidant, anti-inflammatory, and antimicrobial properties of NEG-IS gel against selected oral, fungal and periodontal pathogens?

8. Is NEG-IS cytotoxic on the HGF cell lines?

1.5 Objectives of the study

1.5.1. General objective

To formulate and evaluate the biological properties including antioxidant, anti-inflammatory, and antimicrobial properties of a novel NEG-IS formulation containing *N. sativa* and ginger-based CDs against selected oral pathogens.

1.5.2. Specific objectives

1. To characterise and evaluate the antioxidant, anti-inflammatory, and anti-microbial properties of NSEO against selected oral, fungal, and periodontal pathogens in comparison to a standard drug and positive control, chlorhexidine gluconate 0.2% (CHX 0.2%)

2. To determine and evaluate the cytotoxicity effects of NSEO over HGF cell lines in comparison to CHX 0.2%.

3. To synthesize CDs from ginger by green synthesis method.

4. To characterise and evaluate the physicochemical properties, antioxidant, anti- inflammatory, and antimicrobial properties of ??? against selected oral,

fungal, and periodontal pathogens in comparison to a suitable standard drug and positive control CHX 0.2%.

5. To determine and evaluate the cytotoxicity effects of CDs on HGF cell lines.
6. To formulate nanoemulgel *in-situ* (NEG-IS) that exhibits the optimized concentration of NSEO and CDs.
7. To characterise and evaluate the antioxidant, anti-inflammatory, and antimicrobial properties of NEG-IS against selected oral, fungal and periodontal pathogens in comparison with chlorhexidine gluconate gel 1% (CHX gel).
8. To determine and evaluate the cytotoxicity effects of NEG-IS on HGF cell lines in comparison with CHX gel.

1.6 Null Hypothesis

1. There is no significant difference in the biological properties including antioxidant, anti-inflammatory, and anti-microbial properties against selected oral, fungal, and periodontal pathogens, between NSEO, standard drug and positive control CHX 0.2%.
2. There is no significant difference between NSEO and CHX 0.2% in their cytotoxicity effect on the HGF cell lines.
3. CDs cannot be synthesized from ginger using green synthesis method.
4. There is no significant difference between CDs and the positive control CHX 0.2% in their antioxidant, anti-inflammatory, and anti-microbial properties against selected oral, fungal, and periodontal pathogens.
5. There is no significant difference between CDs and positive control CHX 0.2% in their cytotoxicity effect on HGF cell lines.
6. The formulated NEG-IS does not exhibit the optimized concentration of NSEO and CDs.

7. There is no significant difference between NEG-IS and positive control CHX gel in their antioxidant, anti-inflammatory, and anti-microbial properties against selected oral, fungal, and periodontal pathogens.
8. There is no significant difference between NEG-IS and CHX gel in their cytotoxicity effect on HGF cell lines.

CHAPTER 2

LITERATURE REVIEW

2.1 Periodontal disease

Periodontal disease is a term used to ascribe to some pathological conditions characterized by degeneration and inflammation of gums, periodontal ligaments, alveolar bone, and dental cementum (Garala *et al.,*2013). The detrimental process is linked to the existence of microbial communities located beneath the gingival line and a concentrated infiltration of immune cells and inflammatory substances in the tissues surrounding the teeth, known as the periodontium. If not adequately addressed, this process can result in the loss of teeth. Gingivitis is a type of periodontal disease that is reversible and does not lead to bone loss. In this condition, the inflammatory process is limited to the gingival epithelium and the connective tissue, without impacting the deeper components of the periodontium. Nevertheless, it is important to acknowledge that gingivitis plays a significant role as a primary risk factor and an essential precursor to the development of periodontitis (Hajishengallis *et al.,*2020).

It is vital to note that periodontal diseases are inflammatory and destructive diseases of the dentogingival complex closely linked to the presence of specific periodontal pathogens inhabiting periodontal pockets. While there is a widespread consensus that the disease has a complex origin, empirical evidence indicates that certain gram-negative microbes present in the subgingival plaque biofilm have a significant impact on the onset and advancement of periodontitis. The consortium consisting of *P. gingivalis*, *T. denticola* and *T. forsythia* is commonly found in the subgingival biofilm and is well recognized as the primary group of bacteria responsible for periodontal disease. Other microorganisms include *A. actinomycetemcomitans*, *F. nucleatum*, *P. intermedia*, *Campylobacter rectus*, *Peptostreptococcus migros*, and *Eikenella corrodens* (Popova *et al.,*2013). The onset of periodontitis occurs when the tissues become colonized by pathogenic organisms. The subsequent stage involves the infiltration of bacteria or the infiltration of pathogenic substances into the periodontal tissues, as well as the interactions between bacteria or their substances and host cells. These interactions lead to the direct or indirect deterioration of the periodontium, ultimately leading to tissue degradation (Popova *et al.,*2013).

Red complex pathogens in the bacterial biofilm are most frequently associated with the initiation and progression of periodontitis (Saquib *et al.,*2019). Three different bacterial species, *P. gingivalis*, *T. denticola*, and *T. forsythia,* compose the red complex (Socransky *et al.,*1998). Increased levels of *P. gingivalis*, *T. denticola*, and *T. forsythia*, as well as the red complex (coexistence of all three species at the same lesion), have been seen in cases with stage III and IV periodontitis. As opposed to this, *A. actinomycetemcomitans* is usually associated with stage IV periodontitis of the molar incisor as well as some cases of stages III and IV periodontitis (Tonetti *et al.,*2018; Saquib *et al.,*2019; Zhao *et al.,*2022). HGFs cells play a crucial role in the maintenance of oral homeostasis, as they are the predominant cell type found in the oral cavity and hence their maintenance and protection against any damage is vital (Fadl and Leask, 2023).

2.2 Herbs in Dentistry

Due to their promising outcomes and limited side effects, medicinal herbs and plants are currently attracting increasing interest on a global scale in the treatment of numerous ailments. The World Health Organization (WHO) estimates that 60–80% of the world's population relies on traditional medicine or herbal medicines for their main medical care (WHO, 2004). Owing to their medicinal properties, herbs have been used in dentistry for ages (Cruz Martínez *et al.,*2017) (Table 2.1). They are a natural substitute and have been demonstrated to have fewer adverse effects than other medications such as systemic antibiotics. Aloe vera, chamomile, clove, echinacea, peppermint, and tea tree oil are a few of the plants that are frequently used in dentistry (Taheri *et al.,*2011). Incorporating these herbs into dental care practices can lead to a more holistic approach to oral health and potentially reduce the need for synthetic and invasive treatments. For instance, a study revealed that clove oil was successful in minimizing the discomfort related to dental operations (Shende *et al.,*2016). Aloe vera gel was successful in lowering the severity of gingivitis, according to a different study by Sujatha *et al.,*2014.

Table 2.1. Commonly used medicinal plants for gingival and periodontal diseases (Adapted from Pasupuleti *et al.*,2023; Singh *et al.*,2019)

Family and taxon	Parts used	Periodontal use
[Acoraceae] *Acorus calamus*	Rhizome	Paste of rhizome is applied on gingival tissues to relieve pain
[Amaranthaceae] *Achyranthes aspera*	Bark	Treatment of gingival disorders
[Acanthaceae] *Justicia adhatoda*	Twigs	Twigs are used as toothpicks and to treat pyorrhea
[Amaryllidaceae] *Allium sativum*	Bulb	Paste of the bulb is applied to the gingival tissues to treat infection
[Anacardiaceae] *Anacardium occientale*	Twigs and leaves	Used for routine cleaning of teeth
[Cashews] *Mangifera indica*	Twigs, leaves	Used for regular cleaning of teeth
[Ginger family *Curcuma longa*	Rhizome	To treat pyorrhea and gingival infections
[Leguminosae] *Acacia Arabica*	Tender leaves, bark	Decoction of tender leaves is used as a mouthwash and to treat spongy gums. Burnt bark used as tooth powder
Acacia catechu [Legumes]	Seeds	Reduces bacterial load
[Legumes] *Glycyrrhiza glabra*	Root	Effectively reduces plaque formation by its antibacterial effect
Pongamia pinnata [Legumes]	Leaves	Used for routine cleaning of teeth
[Mahogany] *Azadirachta indica*	Bark	To treat pyorrhea and gingival infections
[Mints] *Vitex negundo*	Leaves	Leaf decoction is used as a mouthwash
[Mulberry family] *Ficus bengalensis*	Latex, twigs	Slender twigs are used as a toothbrush

Table 2.1 Continued

[Myrtle family] *Psidium guajava*	Leaves	Used to treat gingival inflammations and oral ulcers
[Palms] *Areca catechu*	Pericarp	Used for regular cleaning of teeth
[Palms] *Cocos nucifera*	Husk	For regular cleaning of teeth
[Phyllanthaceae] *Emblic myrobalan*	Fruit	General rebuilder of oral health
[Piperaceae] *Piper betle*	Leaves	Used in the treatment of malodor
[Piperaceae] *Piper nigrum*	Fruit	Treatment of oral infection
[Punicaceae] *Punica granatum*	Fruit	Used to treat bleeding gums
[Rose family] *Crateagus oxycanthus*	Fruit	Stabilizes collagen and strengthens the gingival tissues
[Rutaceae] *Citrus limonum Risso*	Outer peel	For routine cleaning of teeth
[Rutaceae] *Citrus medica*	Fruit	Used to treat bleeding gums
[Sapindaceae] *Sapindus mucorosai*	Seed	Used in the treatment of gingival inflammation
[Salvadoraceae] *Salvadora persica*	Stem	Used as a chewing stick for oral care
[Sapotaceae] *Mimusops elengi*	Stem	To treat inflamed gingiva and pyorrhea
[Spurges] *Jatropha curcas*	Stem, leaves	Decoction of leaves used as a mouthwash and to cure bleeding gums. The stem can be used as toothbrush
[spurge family, Euphorbiaceae] *Jatropha gossypifolia*	Twigs	Twigs can be used as toothbrush for routine cleaning
[Spurges] *Ricinus communis*	Root, leaves	Roots are used as a toothbrush. Leaf juice is used as a mouth wash
[Walnut family] *Juglens regi*	Oils, fruit	Used to make tooth powder and to treat pyorrhea
[Ranunculaceae] *N. sativa*	Seeds, oil	Used to treat gingival and plaque inflammation

2.3 *Nigella sativa* as a medicinal herb and its potential in oral health management

Nigella sativa (N. sativa), which has been deemed the "miracle herb of the century," is one of the top-ranked evidence-based herbal remedies (Yimer *et al.,*2019). *N. sativa* has been used by many cultures in Asia, Africa, Europe, the Middle East, and South Asia as a natural remedy for several diseases. The flowering plant *N. sativa* is indigenous to the Mediterranean and southwest Asia. It is referred to by several names, including black cumin in the holy bible, kalonji in the South Asia, habbat al barakah by the Arabs, black seed, and black caraway. It is a herbaceous plant belonging to the family Ranunculaceae and the genus *Nigella*. It is widely cultivated in many parts of the world for its seeds, which are used as a spice and in traditional medicine (Yimer *et al.,*2019).

This plant's seeds have been used for medicinal purposes for ages in systems of conventional medicine like Ayurveda and Unani. Recent research showing the pharmacological effects of *N. sativa* has drawn attention to its therapeutic potential in contemporary medicine (Hannan *et al.,*2021; Iqbal *et al.,*2019). Numerous research have revealed the anti-inflammatory, antioxidant, antibacterial, and immune-modulatory effects of *N. sativa*, making it a promising therapeutic agent for a wide range of illnesses (Guergouri *et al.,*2018; Al-Oqail, *et al.,*2017). It has been demonstrated, for instance, to have positive effects in the treatment of diabetes, hypertension, asthma, allergies, and many cancers. It has been discovered that the active compounds of *N. sativa* provide the herb with its medicinal benefits (Ahmad *et al.,*2021; Ahmad *et al.,*2013; Hannan *et al.,*2021).

2.4 *N. sativa* in the management of periodontal diseases

The use of *N. sativa* has shown promising results in the management of gingivitis and periodontitis. A study demonstrated that *N. sativa* was effective in treating moderate to severe gingivitis. *N. sativa* mouthwash significantly reduced gingival inflammation and dental plaque in patients with moderate to severe gingivitis, as evidenced by lower Gingival Index (GI) and Plaque Index (PI) scores compared to the placebo group, without any significant adverse effects (Singh *et al.,* 2019). Studies have suggested that using an antibacterial agent alongside scaling and root planing is effective for treating periodontal diseases (Bonito *et al.,*2004, Senthilnathan *et al.,*2020). In a study, 20% *N. sativa* was assessed for improving gingival health in comparison to 0.12% chlorhexidine mouthwash. Results indicated that mouthwash containing *N. sativa* was superior for plaque control compared to the standard chlorhexidine. Thus, herbal mouthwash may be a promising alternative for managing gingivitis as an adjunct to the gold standard scaling and root planing (Ilangovan and Rajasekar, 2021).

One study found that a mouthwash containing *N. sativa* extract was effective in reducing gingival inflammation and improving periodontal health in patients with periodontitis (Al-Wafi *et al.,*2014). An *in-vitro* study investigated the antibacterial effects of toothpaste containing *N. sativa* against key pathogens (*P. gingivalis* and *A. actinomycetemcomitans*) associated with periodontitis and its effects on fibroblast cells. The researchers found that 10% *N. sativa* extract, was the most effective concentration for its antimicrobial properties. For cytotoxicity testing, they used human gingival fibroblast cells to determine the safety of *N. sativa* on oral tissues. The findings suggested that *N. sativa* toothpaste had strong antibacterial activity against these pathogens, while being safe for fibroblasts, indicating its potential use as an adjunctive therapy in managing periodontitis (Setiawatie *et al.,*2022).

Senthilnathan *et al.,* (2020) explored the potential of *N. sativa* as a local drug delivery agent for managing periodontitis by evaluating its antibacterial effects against *P. gingivalis* and *P. intermedia*. Methanolic extracts of *N. sativa* seeds were tested at three different concentrations: 5%, 10%, and 15% for their ability to inhibit bacterial growth and were compared to tetracycline, a standard antibiotic used in periodontal therapy. Although the antibacterial activity of *N. sativa* was slightly less effective than tetracycline, all three concentrations showed statistically significant antibacterial activity against the tested periodontal pathogens. The finding suggests that *N. sativa* has promising antibacterial effects against the periodontal pathogens, but further clinical research is needed to confirm its efficacy in patients with periodontitis.

Another study aimed to develop a biodegradable periodontal chip containing thymoquinone—a bioactive constituent of *N. sativa* and to evaluate its effectiveness in managing chronic periodontitis as a local drug delivery agent. Thymoquinone and chitosan were used to formulate the chips. Twelve patients with periodontal pockets measuring ≥ 5 mm participated, resulting in a total of 180 pockets assessed. The mean reductions in probing pocket depth were 0.82 mm for the scaling and root planing group alone, 1 mm for chitosan group used as an adjunct to scaling and root planing, and 1.33 mm for the thymoquinone chip group. The thymoquinone group showed significantly greater improvement than the other groups, with the highest measured gain of 1.33 mm (Al-Bayaty *et al.,*2013).

2.5 Detailed study of *N. sativa*

2.5.1 Extraction methods for *N. sativa* essential oil

Studies have been conducted on the extraction of both types of oils from *N. sativa* seeds using various methods such as hydro-distillation, cold pressing, steam distillation, soxhlet extraction, supercritical fluid extraction and newer methods like microwave-assisted extraction and accelerated solvent extraction. Each method has its advantages and disadvantages, and researchers may choose the method based on the specific properties needed in the oil (Abedi *et al.,*2017) (Table 2.2). However, it is important to note that essential oils are typically more concentrated and potent than volatile oils (Bakkali *et al.,*2008)

Microwave-assisted extraction is a fast and efficient extraction method that allows for the use of small amounts of solvent, which reduces the risk of contamination and saves time. However, it requires specialized equipment and may not be suitable for extracting certain compounds. Accelerated solvent extraction, on the other hand, is a versatile method that can be used for a wide range of samples and has a high reproducibility rate. However, it requires a high initial investment cost and can be time-consuming (Rahim *et al.,*2022).

Hydro-distillation is a common method for extracting *N. sativa* oil. It can produce a higher yield of oil compared to other methods and can be used to extract essential oils from other parts of the plant. However, it may not preserve the natural beneficial compounds in the oil as well as cold pressing. Hydro-distilled oils specifically refer to oils obtained through hydro-distillation, while volatile oils can be extracted using various techniques. Volatile oil is a general term that includes all essential oils regardless of the extraction method, while hydro-distilled oil is specific to the hydro-distillation process (Bakkali *et al.,*2008). Hydro-distilled oils are typically more concentrated and potent than volatile oils, making them ideal for research studies. Volatile oils are natural oils extracted from plants that evaporate readily at room temperature, giving them their characteristic strong aromas. Essential oils are a subset of volatile oils that have been concentrated through processes like distillation or cold pressing, making them extremely potent and rich in the plant's active compounds (Bakkali *et al.,*2008). Research has shown that *N. sativa* oil extracted through hydro-distillation has therapeutic potential, including anti-inflammatory, antioxidant, and antimicrobial properties (Hosseinzadeh *et al.,*2007).

Table 2.2: Extraction techniques for oils - Advantages and Disadvantages (Abedi *et al.*,2017, Rahim *et al.*,2022 and Hosseinzadeh *et al.*,2007)

Extraction Technique	Advantages	Disadvantages
Hydro-distillation	Simple and widely used technique, good for essential oils extraction	Time-consuming May result in the loss of some heat-sensitive compounds
Cold Pressing	No solvents or heat used, preserving the natural composition Environmentally friendly	Lower yield May not extract all the beneficial compounds effectively
Steam Distillation	Common method for extracting essential oils High purity of extracted oils	Long extraction time High energy consumption Possible degradation of heat-sensitive compounds
Soxhlet Extraction	Efficient extraction method for a wide range of compounds High yield	Requires large amounts of solvent Time-consuming Potential risk of thermal degradation
Supercritical Fluid Extraction (SFE)	High efficiency and selectivity Uses non-toxic CO_2 as a solvent Can extract thermally labile compounds	Expensive equipment High operational costs Requires careful optimization of conditions
Microwave-assisted Extraction (MAE)	Fast and efficient Requires small amounts of solvent Reduces the risk of contamination and saves time	Requires specialized equipment May not be suitable for extracting certain compounds
Accelerated Solvent Extraction (ASE)	Versatile and can be used for a wide range of samples High reproducibility rate	High initial investment cost Can be time-consuming

2.5.2 Phytoconstituents' analysis and characterization of *N. sativa* essential oil

2.5.2.(a) Phytochemical screening

Nigella sativa essential oil (NSEO) is known to contain various phytochemical constituents including steroids, triterpenoids, alkaloids, flavonoids, phenols, proteins, tannins, and flavonols. These constituents have been found to exhibit anti-inflammatory, antioxidant, antimicrobial, and anticancer properties (Bolouri *et al.,*2022).

2.5.2.(b) Gas Chromatography Mass Spectrophotometry (GC-MS)

Gas Chromatography Mass spectrophotometry (GC-MS) and gas chromatography-tandem mass spectrophotometry (GC-MS/MS) are two analytical techniques that are used to identify and quantify chemical compounds in complex mixtures. GC-MS involves separating a mixture of compounds by gas chromatography and then ionizing and fragmenting the compounds by mass spectrometry to produce mass spectra that can be used to identify the compounds. It is a powerful technique for identifying and quantifying individual compounds in a mixture. GC-MS/MS, on the other hand, is a variation of GC-MS that uses two mass spectrometers in tandem. The first mass spectrometer separates and ionizes the compounds, and the second mass spectrometer fragments the ions produced by the first mass spectrometer. This allows for even greater sensitivity and specificity in identifying and quantifying compounds in complex mixtures.

A list of bioactive compounds that have been identified by GC-MS/MS analysis of *N. sativa* essential oil with their therapeutic uses is listed in the table 2.3 (Ahmad *et al.,* 2013; Tavakkoli *et al.,*2017).

Table 2.3 A list of bioactive compounds of *Nigella sativa* essential oil and its uses

Compound	Therapeutic Uses
Thymoquinone	Antioxidant, Anti-inflammatory, Anti-cancer, Anti-diabetic, Antimicrobial
Carvacrol	Antimicrobial, Anti-inflammatory, Antioxidant, Analgesic, Anti-cancer
T-anethole	Anti-inflammatory, Antioxidant, Anti-cancer, Anti-ulcer, Anti-anxiety
p-Cymene	Antimicrobial, Anti-inflammatory, Antioxidant, Analgesic
α-Thujene	Anti-inflammatory, Antioxidant, Anti-cancer
α-Pinene	Anti-inflammatory, Anti-cancer, Antimicrobial, Anti-diabetic
β-Pinene	Anti-inflammatory, Anti-cancer, Antimicrobial, Anti-diabetic
Longifolene	Anti-inflammatory, Antioxidant, Antimicrobial, Anti-cancer
Limonene	Anti-inflammatory, Anti-cancer, Antimicrobial, Anti-diabetic
γ-Terpinene	Anti-inflammatory, Antimicrobial, Anti-cancer
Terpinolene	Anti-inflammatory, Antioxidant, Anti-cancer
α-Phellandrene	Anti-inflammatory, Antioxidant, Anti-cancer
Sabinene	Anti-inflammatory, Anti-cancer, Antimicrobial, Anti-diabetic
Myristicin	Anti-inflammatory, Analgesic, Anti-cancer
Thymohydroquinone	Antioxidant, Anti-inflammatory
Iso-thymohydroquino ne	Antioxidant, Anti-inflammatory
Nigellidine	Anti-inflammatory, Antioxidant, Anti-cancer
Nigellimine	Anti-inflammatory, Antioxidant, Anti-cancer
Nigellimine-N-oxide	Anti-inflammatory, Antioxidant, Anti-cancer
Nigellidine-N-oxide	Anti-inflammatory, Antioxidant, Anti-cancer