SYNERGISTIC EFFECT OF PEPPERMINT OIL AND BLACK SEED OIL LOADED INTO ALGINATE BEADS FOR TREATING IRRITABLE BOWEL SYNDROME IN ANIMAL MODEL

BY

MD. ABUL KALAM AZAD

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Kulliyyah of Pharmacy
International Islamic University Malaysia

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ABSTRACT

Black seed oil (BSO) have traditional claims and scientific evidences for therapeutic and pharmacological values. Similarly, peppermint oil (PO) has been used for a long time as a therapeutic agent for stomach related diseases. In conjunction with their therapeutic benefit, they have become an interest in utilization in pharmaceutical use. The key objective and major challenge of this study was to develop a pH sensitive BSO and PO loaded alginate bead as an intestinal release matrix system designed to release in the intestine without releasing the drug in the gastric fluid. To overcome these challenges, organic-solvent-free, green, and environmentally friendly electrohydrodynamic atomization (EHDA) technique was employed to prepare BSO and PO loaded alginate beads. This process enabled the formulation of small size and uniform beads with suitable diffusion, and swelling characteristic resulting in process performance enhancement. The current study deals with the development, optimization, and *in vitro* characterization of BSO and PO beads in different aspects like emulsion stability (ES), particle size distribution, zeta potential, yield percentage (Y%), physical appearance i.e. scanning electron microscopy (SEM), encapsulation efficiency (EE%), quantification of thymoquinone and menthol, shape, weight uniformity, *ex vivo* mucoadhesive properties, *in vitro* drug release profile, and gastrointestinal tract (GIT) beads distribution. Prior to that, the compatibility was tested using attenuated total reflectance-Fourier-transform infrared spectroscopy (ATR-FTIR) and differential scanning calorimetry (DSC). Then the optimized formulation was administered to evaluate the therapeutic efficacy as an anti-inflammatory effect of BSO and PO loaded beads in irritable bowel syndrome (IBS) in mustard oil (MO)-induced Sprague-Dawley rats. The results indicate that the voltage and flow rate have significant influenced on beads size and sphericity factor as well as on encapsulation efficiency. All prepared formulations (F1-F9) exhibited low release rate in simulated gastric fluid (SGF) (pH 1.2) within 2 h. However, all these beads (F1-F9) showed better drug release profile in simulated intestinal fluid (SIF) (pH 6.8) at the next 2 h. The optimized formulation (F8) has shown excellent *ex-vivo* mucoadhesive properties, well distributed in various parts of the intestine, well swelling behaviour and release in the SIF. BSO and PO-loaded alginate beads exhibited potential improvement of IBS on MO-induced rat compare to non-treatment group. These formulations were significantly suppressed proinflammatory cytokines like interleukin- IL-1β, IL-6, and TNF-α expression upregulated of anti-inflammatory cytokine (IL-10) expression in MO-induced intestinal inflammation. However, within the treatment groups, BSO-loaded alginate beads potentially upregulated the anti-inflammatory cytokine (IL-10) expression compared to other treatment groups. The combination of BSO (75 mg) and PO (25 mg) treatment group showed synergistic therapeutic effect by improving disease symptoms and suppressing IL-1β, IL-6, and TNF-α expression. The technique for the preparation of beads was found to be simple, reproducible, easily controllable, economical, and appeared to be a promising approach to control the bead's nature and to ensure release into targeted site after oral administration. This formulation is considered as an anti-inflammatory drug candidate with possible synergistic effect for IBS treatment.

Keywords: black seed oil, Peppermint oil, electrohydrodynamic technique, microencapsulation, inflammation, IBS.

خلاصة البحث

للحبة السوداء وزيتها ادعاء تقليدي ودليل علمي للقيمة العلاجية والدوائية. وقد تم استخدامهما لعدة قرون لعلاج أنواع مختلفة من الأمراض في الإنسان. وبالمثل ، فإن زيت النعناع (PO) قد استخدم لفترة طويلة لعلاج أمراض المعدة. بالاقتران مع فائدتهما العلاجية ، فقد أصبحوا محط اهتمام للاستخدام على شكل مستحضر صيدلاني. كان الهدف الرئيسي والتحدي الرئيسي للدراسة هو تطوير حبة ألجينات محملة بـ زيت الحبة السوداء و زيت النعناع كنظام إطلاق طويل المدى في سوائل الأمعاء مصمم للبقاء في المعدة دون إطلاق الدواء في سائل المعدة. للتغلب على هذه التحديات ، تم استخدام تقنية الانحلال الكهروهيدروديناميكي الخالية من المذيبات العضوية والصديقة للبيئة لتحضير حبات الجينات المحملة بـزيتي النعناع والحبة السوداء. تتيح هذه العملية صياغة حبيبات صغيرة ومتماثلة الحجم وانتشار مناسب وخصائص انتفاخ تؤدي إلى تحسين أداء عملية اطلاق الدواء. تتناول الدراسة الحالية التطوير والتحسين والتوصيف في المختبر في جوانب مختلفة مثل ثبات المستحلب وتباين حجم الحبيبات وفرق جهد زيتا ونسبة الناتج ومظهر الحبيبات وكفاء الاحتواء داخل حبيبات المستحلب والقياس الكمي للمنثول، و كذلك الشكل ، و توحيد الوزن ، و خصائص اللصق المخاطي خارج الجسم الحي ، وإطلاق الدواء في المختبر ، وانتشار الحبيبات في الجهاز الهضمي.. قبل ذلك ، تم اختبار التوافق بين مكونات المستحضر باستخدام التحليل الطيفي للأشعة تحت الحمراء والمسح التفاضلي. ثم تم إعطاء الصيغة المحسنة لتقييم الفعالية العلاجية كتأثير مضاد للالتهابات من حبات زيت الحبة السوداء والنعناع في متلازمة القولون العصبي في فئران سبراج داولي المستحثة بزيت الخردل. أشارت النتائج إلى أن الجهد ومعدل التدفق لهما تأثير معنوي على حجم الحبيبات وعامل كروية وكذلك على كفاءة التغليف. كل هذه التركيبات (F1-F9) وجدت معدل إطلاق منخفض جدًا في محاكاة السائل المعدي (الرقم الهيدروجيني 1.2) عند ساعتين. ومع ذلك ، أظهرت كل هذه التركيبات (F1-F9) معدل اطلاق أفضل في السائل المعوي المحاكي (الرقم الهيدروجيني 6.8) في الساعتين التاليتين. أظهرت التركيبة المُحسَّنة (F8) خصائص لاصق مخاطي وانتفاخ ممتازة خارج الجسم الحي وفي وسط الأمعاء. تم العثور على الحبيبات موزعة بشكل جيد في أجزاء مختلفة من الأمعاء في دراسة في الجسم الحي. أظهرت حبيبات الالجينات المحملة بـ زيت النعناع تحسنًا محتملًا في التهابات أمعاء الفئران التي يسببها زيت الخردل مقارنة بالمجموعة غير المعالجة. نرجح هذه التركيبة لتكون دواء مضاد للالتهابات بالإضافة إلى تأثير تآزري محتمل ، والذي يمكن أن يثبط بشكل كبير افراز السيتوكين المسبّب للالتهابات في التهاب الأمعاء. من بين المجموعات العلاجية تبين أن المعالجة بالخرزات المحتوية على زيت الحبة السوداء سبب ارتفاعاً ببتراكيز السايتوكين المضاد للالتهاب (IL-10). أظهرت مجموعة المشاركة بين زيت الحبة السوداء (75 mg) وزيت النعناع (25 mg) تأثيرا تسانديا لتحسين الأعراض وتثبيط تعبير IL-1β, IL-6 وTNF-α . تعتبر تقنية تحضير حبات الألجينات التي تحتوي على زيت الخردل والنعاع بسيطة وقابلة للتكرار ويمكن التحكم فيها بسهولة واقتصادية ومتسقة ويبدو أنها نهج واعد وموثوق به للتحكم في طبيعة الحبيبة ولضمان إطلاق الدواء بعد تناولها عن طريق الفم. علاوة على ذلك ، يمكن أن تكون الخرزات المحسّنة دواءً محتملاً لعلاج القولون العصبي بنجاح.

الكلمات المفتاحية: زيت الحبة السوداء ، زيت النعناع ، التقنية الكهرومائية ، الكبسلة الدقيقة ، الالتهابات، متلازمة الكولون المتهيج.

APPROVAL PAGE

The thesis of Md. Abul Kalam Azad has been approved by the following:

_____
Abd Almonem Doolaanea
Supervisor

_____
Bappaditya Chatterjee
Chairman of Supervisory Committee

_____
Wan Mohd. Azizi
Member of Supervisory Committee

_____
Suzanah Abdul Rahman
Internal Examiner

_____
Haliza Katas
External Examiner

_____
Yusrida Darwis
External Examiner

_____
Zarina Zainuddin
Chairman

DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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LIST OF ABBREVIATIONS

IBS	Irritable Bowel Syndrome
IFFGD	The International Foundation For Functional Gastrointestinal Disorders
IBS-C	Constipation Predominant Irritable Bowel Syndrome
IBS-D	Diarrhoea Predominant Irritable Bowel Syndrome
IBS-M	Mixed Irritable Bowel Syndrome
GI	Gastrointestinal
GIT	Gastrointestinal Tract
FDA	U.S. Food and Drug Administration
Y%	Percentage Yield
SF	Sphericity Factor
SEM	Scanning Electron Microscope
ATR-FTIR	Attenuated Total Reflection- Fourier Transform Infrared
EE%	Percentage of Encapsulation Efficiency
GC-FID	Gas Chromatography-Flame-Ionization Detection
MO	Mustard Oil
TQ	Thymoquinone
TNF-α	Tumour Necrosis Factor-A
IFN-γ	Interferon-Gamma
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
PGE2	Prostaglandin E2
WGO	World Gastroenterology Organisation
AGA	American Gastroenterological Association
IMMCs	Intestinal Mucosal Mast Cells
mMCP-1	Mouse Mast Cells Protease-1
PAF	Platelet-Activating Factor
MDA	Malondialdehyde
NO	Nitric Oxide
GPX	Glutathione Peroxidase
LTB4	Leukotriene B4
5-LO	Synthase 5-Lipoxygenase
LTs	Leukotrienes
LTC4	Leukotriene C4
SN	Sodium Nitrite
MAPKs	Mitogen Activated Protein Kinases
NF-κB	Nuclear Factor Kappa B
TLR	Toll-Like Receptor
TGF-β1	Transforming Growth Factor- B1
RT-PCR	Reverse Transcription Polymerase Chain Reaction
ApoE	Apolipoprotein E
PDA	Pancreatic Ductal Adenocarcinoma

MCP-1	Monocyte Chemoattractant Protein-1
CFB	Complement Factor B
COX-1	Cyclooxygenase 1
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
PI3K	Phosphoinositide 3-Kinase
LPS	Lipopolysaccharide
HNE	4-Hydroxy-Trans-2-Nonenal
OVA	Ovalbumin
Pi-IBS	Post-Infectious Irritable Bowel Syndrome
CDR	Cumulative Drug Release
PEG	Polyethylene Glycol
TCAs	Tricyclic Antidepressants
NaOH	Sodium Hydroxide
HCI	Hydrochloric Acid
N. sativa	*Nigella Sativa*
EI	Enteric Infection
EC	Enterochromaffin
DCA	Deoxycholic Acid
DSS	Dextran Sulfate Sodium
ROS	Reactive Oxygen Species
TNBS	Trinitrobenzene Sulfonic Acid
S	Sensitivity
P	Permeability
H	Histopathology
SE	Secretion
PA	Pathogenesis
H	Visceral Hypersensitivity
M	Motility Dysfunction
S	Secretion Alterations
P	Permeability Alteration
ICH	International Conference on Harmonization
HPLC	High-Performance Liquid Chromatography
W/V	Weight/Volume
PBS	Phosphate Buffer Saline
RSD	Relative Standard Deviation
DL	Detection Limit
QL	Quantification Limit
EDTA	Ethylenediamine Tetraacetic Acid
EHDA	Electrohydrodynamic Atomisation
DSC	Differential Scanning Calorimetry
XRD	X-Ray Diffraction
NSO	*Nigella Sativa* Oil
H. Pylori	*Helicobacter Pylori*
ES	Emulsion Stability
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
ANOVA	Analysis of Variance
SD	Standard Deviation
PDI	Polydispersity Index

API	Active Pharmaceutical Ingredient
CaCl2	Calcium Chloride
DE	Dissolution Efficiency
MDT	Mean Dissolution Time
IG	Ionic Gelation
BC	Before Centrifuge
AC	After Centrifuge
USP	United States Pharmacopeia
MLCK	Myosin Light Chain Kinase
IIUM	International Islamic University Malaysia
DAI	Disease Activity Index
NC	Negative Control
DC	Drug Control
PO	PO Treatment
BSO	BSO Treatment
Comb	Combination Treatment
W	Week
ME	Muscularis Externa
SM	Submucosal
M	Mucosal
BG	Brunner's Gland
V	Villi
NSAID	Nonsteroidal Anti-Inflammatory Drug
Th1	T Helper Cell Type 1

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

According to the report of gastroenterologists, irritable bowel syndrome (IBS) is one of the most widely recognized ailments, which is responsible to diminish quality of daily life and socioeconomic burden significantly (Camilleri M, 2001). IBS has a significant occurrence up to 10–15% of population worldwide reported by the International Foundation for Functional GI Disorders (IFFGD, 2016). Among all IBS patients, nearly 25% patients are suffering from severe IBS which is lower in rate than patients experiencing moderate and mild IBS as per reported 35% and 40% respectively. In the USA, about 2.4 to 3.5 million people visits physicians because of IBS annually. According to them, a cost of nearly $21 billion is estimated per year due to IBS by means of direct and indirect treatment expenditures as well as loss of productivity and work absenteeism. In the community, there are significant proportions of individuals' female approximately 60% to 65% report IBS, on the other hand, male who reports IBS almost 35% to 40% of individuals (Canavan, west & Card, 2014; IFFGD, 2016). A brief description of symptoms of IBS has been summarised in Table 1.1.

Table 1.1 IBS symptoms summarised based on diagnostic criteria

Diagnostic criteria	**Symptoms, signs, and laboratory investigations included in criteria**
Manning (1978)	• Abdominal pain relieved by defecation • More frequent stools with onset of pain • Looser stools with onset of pain • Mucus per rectum • Feeling of incomplete emptying • Patient-reported visible abdominal distension
Kruis (1984)	• Abdominal pain, flatulence, or bowel irregularity • Description of character and severity of abdominal pain • Alternating constipation and diarrhea • Signs that exclude IBS (each determined by the physician): • Abnormal physical findings and/or history pathognomonic for any diagnosis other than IBS • Erythrocyte sedimentation rate > 20 mm/2 h • Leukocytosis > 10000/cc • Anemia (Hemoglobin < 12 for women or < 14 for men) • Impression by the physician that the patient has rectal bleeding
Rome I (1990)	• Abdominal pain or discomfort relieved with defecation, or associated with a change in stool frequency or consistency, • Plus, two or more of the following on at least 25% of occasions or days for 3: • Altered stool frequency • Altered stool form • Altered stool passage • Passage of mucus • Bloating or distension
Rome II (1999)	• Abdominal discomfort or pain that has two of three features for 12 wk (need not be consecutive) in the last one year: • Relieved with defecation • Onset associated with a change in frequency of stool • Onset associated with a change in form of stool
Rome III (2006)	• Recurrent abdominal pain or discomfort three days per month in the last 3 mo associated with two or more of: • Improvement with defecation • Onset associated with a change in frequency of stool • Onset associated with a change in form of stool

It is being used for centuries to treat various types of disorders in human. Thymoquinone (TQ) is referred as a significant phytochemical ingredient of BSO.

Agbaria et al. (2015) has been reported for wide spectrum of pharmacological properties of TQ which has been included *in vitro* and *in vivo* studies.

Similarly, peppermint has been used for a long time as a treatment agent for stomach related diseases. PO has particularly been assessed for IBS treatment effectively for many years. PO as well as its principle active components, l-menthol is very well known for providing smooth muscle Ca++ channel antagonism, orocecal transit time normalization, carminative actions, kappa opioid agonism, anti-infective activities and anti-inflammatory activities and serotonergic antagonism (5HT3) (Cas, Epstein & Shah, 2016). All proposed possible mechanisms of action attract attention to PO as a therapeutic agent for IBS.

The combination of alginate beads microparticles of BSO and PO formulation developed to provide delayed delivery of active ingredient into the small intestine. The purposes of the study are to delineate the safety, effectiveness, tolerability and synergic effect of this innovative pharmaceutical preparation of BSO and PO to treat the worldwide spread of GI symptoms in individual patient with IBS.

1.2 STATEMENT OF THE PROBLEM

IBS and its related complications cause noteworthy public health care burden to the society. There are no prescriptions to treatment this ailment till now due to not discover the underlying pathophysiology of IBS. The most generally implemented treatment for the attenuation of symptomatic mild to severe IBS includes prokinetics and antispasmodics drug for the patients with constipation-predominant irritable bowel syndrome (IBS-C) and diarrhoea-predominant irritable bowel syndrome (IBS-D). Besides, opioid agonists (diphenoxylate and loperamide), anticholinergics, and 5-HT3 (Tegaserod, ondansetron and granisetron) have been widely used for the treatment of

IBS C & D. However, the use of these drugs has been declined due to an expanded risk of cardiovascular disorder, hyposalivation and reduce in GI motility. There are different types of anti-infective gut-specific rifaximin, nystatin and tetracycline being tested by IBS patients but unfortunately, they have showed unexpected systemic adverse effects with very less response rate.

The present pharmacological management strategies emphasize on minimizing severity of the symptoms while frequently the quality of life is declining due to considerable side effects of present accessible remedies. This has prompted a usefulness opening for IBS patients who look for help to upgrade their personal quality of life. BSO and PO have been reported to contribute in the management of IBS symptoms to higher degree and personal satisfaction of patient's daily life as well, but either PO or BSO has been used separately, no combined use has been reported. There is no such matrix to deliver the drug to specific site for inflammatory bowel syndrome. Therefore, it has become very necessary to develop a drug carrier to deliver active compound into specific site like intestine. In this study, a novel delayed release formulation combining both BSO and PO were developed to target the release in intestine. Moreover, the safety and effectiveness of the formulation to relief IBS symptoms was evaluated in animal model.

1.3 RESEARCH OBJECTIVES

To develop a formulation combining BSO and PO as an oral dosage form and to determine the synergic effect of the combination formulation in IBS. The study aimed to obtain the following objectives:

1- To develop and characterize the alginate bead formulations of BSO.