STUDIES ON THE EXTRACTS OF BLACK CUMIN (*Nigella sativa* L.) OBTAINED BY SUPERCRITICAL FLUID CARBON DIOXIDE

The thesis submitted to the

UNIVERSITY OF MYSORE

In fulfillment of the requirements for the degree of

Doctor of Philosophy
In
BIOTECHNOLOGY

By

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CERTIFICATE

I, **T. V. Suresh Kumar**, certify that this thesis is the result of research work done by me under the supervision of **Dr. K. Udaya Sankar, Chief Scientist** at Food Engineering Department, **Central Food Technological Research Institute, (Council of Scientific and Industrial Research),** Mysore-570 020, India. I am submitting this thesis for possible award of **Doctor of Philosophy (Ph.D.) degree in Biotechnology** of the University of Mysore.

I, further certify that this thesis has not been submitted by me for award of any other degree/diploma of this or any other University.

Signature of Doctoral candidate

Signed by me on……(date)

Signature of Guide *Date:*

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I, hereby certify that the thesis entitled, **"Studies on the extracts of black cumin (*Nigella sativa* L.) obtained by supercritical fluid carbon dioxide"** submitted by **Mr. T. V. Suresh Kumar** to the University of Mysore for the award of the degree of **Doctor of Philosophy in Biotechnology** is the result of research work carried out by him in the Food Engineering Department, Central Food Technological Research Institute (Council of Scientific and Industrial Research), Mysore under my guidance during the period of 2008-2011.

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DECLARATION

I, hereby declare that the thesis entitled **"Studies on the extracts of black cumin (*Nigella sativa* L.) obtained by supercritical fluid carbon dioxide"** which is submitted herewith for the degree of **Doctor of Philosophy in Biotechnology** of the University of Mysore, is the result of the research work carried out by me under the guidance of **Dr. K. Udaya Sankar**, Chief Scientist, Food Engineering Department, Central Food Technological Research Institute (Council of Scientific and Industrial Research), Mysore during the period of 2008-2011.

I further declare that the results of this work have not been previously submitted for award of any other degree/fellowship of this or any other University.

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(T. V. Suresh Kumar)

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABBREVIATIONS AND SYMBOLS

AU	Activity Units
ATCC	American Type Culture Collection
ANOVA	Analysis of variance
α	Alpha
amu	Atomic mass unit
β	Beta
BHI	Brain heart infusion agar
BHA	Butylated hydroxy anisole
BHT	Butylated hydroxy toluene
^{13}C	Carbon-13
CRAB	Carbapenam Resistant *Acenitobacter baumanii*
cm	Centimeter
CAS	Chemical Abstract Service
δ	Chemical shift value
CFU	Colony-forming units
J	Coupling constant
Pc	Critical pressure
Tc	Critical temperature
°C	Degree centigrade
DMSO-d_6	Deuteriated Dimethyl sulfoxide
dia	Diameter
DAEC	Diffusely adherent *Escherichia coli*
DMF	Dimethyl formamide
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
DTQ	Dithymoquinone
d	Doublet
eV	Electronvolt
ESI	Electro Spray Ionization
EAggEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*

EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
EC	Enzyme Commission
E. coli	*Escherichia coli*
RI_{exp}	Experimental retention indices
ESBL	Extended-Spectrum Beta- Lactamase
	Klebsiella pneumoniae ATCC strain (700603)
$FeCl_3$. $6H_2O$	Ferric chloride
FRAP	Ferric Reducing Antioxidant Power
Fig.	Figure
FID	Flame Ionization Detector
FT-IR	Fourier Transform Infra-Red spectroscopy
GAE	Gallic Acid Equivalents
γ	Gamma
GC	Gas Chromatography
GRAS	Generally Regarded As Safe
g	Gram
HAART	Highly active anti-retroviral therapy
HPLC	High performance liquid chromatography
h	Hour
HD $SCCO_2$	Hydrodistillation of Supercritical CO_2
Trolox	6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid
IC 50 values	Inhibitory Concentration for 50% reduction
i	Interchangeable
i.d	Internal diameter
IUPAC	International Union of Pure and Applied Chemistry
kB	Kilo Bite
kDa	Kilo Dalton
kV	Kilovolts
L	Liter
RI_{lit}	Literature retention indices
m/z	Mass / ion charge

MS	Mass Spectrometry/ Mass spectra
MHz	Mega hertz
MPa	Megapascal
mp	Melting point
mRNA	Messenger Ribo Nucleic Acid
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin sensitive *Staphylococcus aureus*
MTCC	Microbial Type Culture Collection
µg	Microgram
µL	Microlitre
µm	Micrometer
µM	Micromolar
µmol	Micromole
µs	Microsecond
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
mg	Milligram
mL/ ml	Milliliter
mm	Millimeter
mM	Millimolar
mmol	Millimole
min	Minute
ε	Molar extinction coefficient
$[M]^+$	Molecular ion
MHA	Mueller–Hinton Agar
m	Multiplet
NIST	National Institute of Standards and Technology
pH	Negative logarithm of hydrogen ion concentration (power of hydrogen)
NSO	*Nigella sativa* oil
N	Normality
NA	Nutrient agar

NBGP	Nutrient broth containing 0.05% phenol red and supplemented with 10% glucose
ODC	Ozone-depleting compounds
ppm	Parts per million
%	Percentage
π	Pi
KBr	Potassium bromide
psi	Pounds per square inch
P value	Probability value
PIs	Protease inhibitors
^{1}H	Proton
Q-TOF	Quadrupole-Time of Flight
ROS	Reactive oxygen species
RSM	Response surface methodology
Rf	Retention factor
RI	Retention Indices
RP-HPLC-DAD	Reversed phase HPLC diode array detector
sec	Seconds
STEC	Shiga-like toxin-producing *Escherichia coli*
Silica gel-G	Silica gel-Gypsum
s	Singlet
Na_2CO_3	Sodium carbonate
SD	Standard deviation
$SCCO_2$	Supercritical carbon dioxide
SFE	Supercritical fluid extraction
SCF	Super critical fluid
SOD	Superoxide dismutase
TBH	Tertiary butylated hydroquinone
TMS	Tetra methyl silane
TLC	Thin layer chromatography
THQ	Thymohydroquinone
THY	Thymol
TQ	Thymoquinone

TPC	Total phenolic content
tr	Trace
TPTZ	2, 4, 6-tripyridyl-s-triazine
TE	Trolox equivalents
2D HSQCT NMR	Two-Dimensional Heteronuclear Single Quantum Coherence Transfer Nuclear Magnetic Resonance
UV	Ultra violet
VTEC	Vero cytotoxigenic *Escherichia coli*
VOC	Volatile organic compounds
V	Volt
v/v	volume by volume
cm^{-1}	Wave per centimeter
λ_{max}	Wavelength of maxima absorbance (lambda max)
w/w	Weight by weight

ABSTRACT

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The pharmacologically valued black cumin seeds of *Nigella sativa* L. plant of Ranunculaceae family are used in folk medicine, as they contain a complex of more than hundred phytochemicals, some of which are yet to be identified, as they are highly unstable. Of the major quinonic phenol compounds thymoquinone (TQ), dithymoquinone (DTQ), thymohydroquinone (THQ) and thymol (THY) present in the seed as bioactives, whereas thymoquinone is the most pharmacologically active compound. Conventional Soxtec™ and supercritical carbon dioxide ($SCCO_2$) extractions were carried out for the seeds. Response surface methodology (RSM) along with Box-Behnken experimental design was employed to maximise the extract yield and TQ content by varying the temperature, pressure and material in $SCCO_2$ extraction. The $SCCO_2$ extraction at optimized conditions (120 bar pressure, 40°C temperature and solvent to material ratio of 45kg/kg) showed higher TQ content and lower of extract yield. $SCCO_2$-1 extract as major volatile part (at 120 bar pressure with 40°C) and total extract obtained as $SCCO_2$-2 (at 280 bar pressure with 50°C) and HD $SCCO_2$ (Hydrodistillation of $SCCO_2$-2) were chemically characterized to report 47 compounds. Of these compounds, 16 have been reported for the first time in *Nigella sativa* seed. The occurrence of four major quinonic phenol compounds; TQ, DTQ, THQ and THY in $SCCO_2$-2 extract was confirmed by 2D HSQCT NMR. Further, the major bioactive compound TQ was isolated and characterized from the $SCCO_2$-2 extract. The $SCCO_2$ extracts showed higher antibacterial activities than Soxtec™ extracts. The isolated TQ showed lower MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values against the major food borne bacteria used in this study and proved to be an antibacterial principle. Antioxidant activities were evaluated by DPPH and FRAP assays with

total phenolic content (TPC), where also $SCCO_2$ extracts indicated higher antioxidant activity than Soxtec™ extracts. The isolated TQ from $SCCO_2$ extract proved with higher antioxidant properties and higher TPC. The synthesis of water soluble thymol glycosides was carried out using amyloglucosidase (from *Rhizopus* mold) and thymol with different carbohydrates in DMF (dimethyl formamide) under $SCCO_2$ conditions. Thymol formed glycosides with D-galactose, D-mannose, D-fructose, D-ribose and D-arabinose. Spectral characterization studies revealed that the reaction occurred between the phenolic OH group of thymol and 1-*O*/2-*O* groups of D-fructose and C-1 group of D-galactose, D-mannose, D-ribose and D-arabinose resulting in monoglycosylated/arylated derivatives. Of the glycosides synthesized, thymol-6-*O*-D-galactopyranoside (α and β forms), thymol-6-*O*-D-mannopyranoside (α and β forms), thymol-6-*O*-2-*O*-D-fructofuranoside and thymol-6-*O*-1-*O*-D-fructofuranoside, thymol-6-*O*-D-ribofuranoside (α and β forms) and thymol-6-*O*-D-arabinofuranoside (α and β forms) were reported for the first time. The synthesized thymol glycosides have showed better bioavailability and pharmacological properties for future applications. Hence, TQ appears to be promising as a potent natural antibacterial as well as an antioxidant principle, which could replace the synthetic antibiotics and antioxidants in food and pharmacological applications.

CHAPTER 1

Introduction

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1.1. Introduction

Most of the commercially and clinically practiced antimicrobials and antioxidants are purely of synthetic origin. The usage of such synthetic chemicals is restricted in many countries, owing to their undesirable and long term toxicological side effects like carcinogenicity. The safety of synthetic antioxidants has also been questioned in recent years (Duh *et al.* 1992). Hence, scientific interest in medicinal plants has burgeoned due to increasing concerns about the side effects of conventional medicines, growing interest in natural products and increased efficiency of new plant-derived drugs. At this juncture, it is highly imperative to find out antimicrobials and antioxidants from natural sources for risk-free food and pharmacological applications.

Phytochemicals

Phytochemicals are natural bioactive compounds, found in plants that work with nutrients and dietary fibers, to protect mankind from diseases. More than a thousand phytochemicals such as polyphenols, carotenoids, glucosinolates, phytates, saponins, amines, or alkaloids have been identified from plant sources. Some of these compounds may contribute to explain the beneficial health effects while consuming fruits and vegetables or whole grain cereals. These are non-nutritive plant chemicals having protective or disease preventing properties in plants. The term 'phytochemical' refers to those plant chemicals that have health related effects on humans and are biologically more active in their near natural forms; nevertheless they are not essential for the human body to survive.

Most of the phytochemicals are organic compounds which are heat labile, volatile, photo reactive and unstable. Though thousands of phytochemicals have been

reported so far, only a fraction of them has been reported and documented. Since 1970, the increasing numbers of research findings have indicated that there is a close relation between what people eat and their health. Hundreds of research reports around the world have shown that diets rich in plant-based foods are associated with lower rates of heart disease and cancer incidence. Some of the commonly known phytochemicals include β-carotene, ascorbic acid (Vitamin C), folic acid and Vitamin E. Terpenes are probably the most common phytochemicals and which is known to help in preventing certain types of cancer and cardiac ailments (Johnson and Williamson 2003).

Activities of phytochemicals

***Antioxidant activity*:** Most of the phytochemicals have antioxidant activities protecting our cells from free radical damage (oxidative stress), thereby reducing the risk of developing certain types of cancer. These antioxidants include allyl sulphides (in onions, leeks and garlic), carotenoids (in fruits and carrots), flavonoids (in fruits and vegetables), and polyphenols (in tea and grape skins). The major polyphenolic compounds in berries are anthocyanins, hydrolysable tannins (gallo-and ellagitannins), flavonols, and flavan-3-ols, including proanthocyanidins (Mertz *et al*. 2007; 2009). These polyphenolics are also of interest in human nutrition and medicines, because of their potent antioxidant capacity and health-protective effect against chronic diseases, including cardiovascular complaints and cancer (Prior and Gu 2005; Dai *et al*. 2009).

***Hormonal action*:** Isoflavones can help to modulate human endocrine system and reduce the menopausal symptoms and osteoporosis. Soybeans and soy products are the main source of isoflavones. Soy isoflavones are included into the so-called

phytoestrogens due to their weak estrogen activity having a potential protective effect (Penalvo *et al*. 2004) against certain types of hormone-dependent ailments like colon, breast and prostate cancers compared to the Western populations (Adlercreutz 1995; Holt 1998; Setchell 1998; Yin *et al*. 2004).

***Stimulation of enzymes*:** Phytochemicals that stimulate enzymes include protease inhibitors (soy and beans), terpenes (citrus and cherry fruits). Some phytochemicals like indoles stimulate enzymes, which make oestrogen less effective and thereby reduce the risk of breast cancer.

***Interference of DNA replication*:** Saponins found in beans can play a role in preventing the multiplication of cancer cells by interfering in the replication of cell DNA.

***Antimicrobial effect*:** Allicin present in garlic has anti-bacterial properties. A bioactive alkaloid berberine is a phytochemical obtained from Croatian barberry expresses antimicrobial activity against different pathogens (Kosalec *et al*. 2009).

Foods that are rich in phytochemicals are: whole grains, vegetables, fruits, and herbs. The easiest way to ensure optimal dosage of phytochemicals is to eat plenty of fresh organic whole foods (http://www.organicfooddirectory.com.au/organic-food/food/phytochemicals.html).

Plant kingdom has always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them. Non conventional oil seeds are being considered, because their constituents have unique chemical properties and may augment the supply of edible oils. Interest in newer sources of seed oils has recently grown. A trend toward the use of natural additives in foods has been apparent for quite some time as a result of consumer demand. Recent

researches have focused on isolation and characterization of effective natural phytochemicals. However, very little information is currently available on the phytochemicals present in crude seed oils that are responsible for their biological activities. They can be used in the food industry, so that their beneficial effects can be exploited within the human body. Crude oils are consumed in their natural state, thus conserving a number of minor substances, which are usually removed during the process of refining (Koski et al. 2002; Tasioula-Margari and Okogeri 2001).

Among the various medicinal plants, black cumin (*Nigella sativa* L.) is of particular interest, because it may be utilized for the production of formulations containing phytochemicals with significant biological properties and health benefits (Salem 2005).

Black cumin (*Nigella sativa* L.)

Nigella sativa L. is an indigenous annual herbaceous dicotyledonous plant that is more commonly known as the fennel flower plant and belongs to the Buttercup or Ranunculaceae family. It is widely grown in the Mediterranean countries, Middle East, Eastern Europe and Western Asia. This plant grows to a maximum height of about 60 cm and has finely divided foliage and blue flowers (Fig 1.1) from which small caraway-type black seeds are produced. The seeds, generally of small size (1–5 mm), with corrugated integuments, represent the useful product (Fig 1.2). The seed has been used for thousands of years as a spice as well as a food preservative. In the Middle East, Northern Africa and India, the seeds are traditionally used for centuries for treating ailments like asthma, cough, bronchitis, headache, rheumatism, fever, influenza and eczema owing to their antihistaminic, antidiabetic and anti-inflammatory properties (Burits and Bucar 2000).

Fig. 1.1. The plant and flower of *Nigella sativa* L.

Taxonomy of *Nigella sativa* L. plant:

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Ranunculales

Family : Ranunculaceae

Genus : *Nigella*

Species : *sativa*

Vernacular names of *Nigella sativa* L. seed

The *Nigella sativa* seed has various vernacular names listed below.

Black cumin (English); Black seed (English); Black-caraway seed (U.S.A.); Habba-tu sawda (Arabic); Karun jiragam (Tamil); Karunshiragam (Malayalam); Karijirigi (Kannada); Nulajirakara (Telugu); Kalanjire (Marathi); Kalonji (Urdu and Hindi); Krishnajirika (Sanskrit); Kalajira (Bengali) and Shonaiz (Persian) (Akram khan 1999).

Fig. 1.2. The seeds of *Nigella sativa* L.

Significance of *Nigella sativa* L. seed

To a wide variety of Persian foods such as bread, yoghurt, pickles, sauces and salads the seeds serve as a spice (Hajhashemi *et al.* 2004). Despite the availability and usage of numerous antiepileptic drugs, nearly 15% of childhood epilepsy cases are treatment-resistant. However, in traditional medicine, *Nigella sativa* has been known for its anticonvulsant effects. In a double-blinded crossover clinical trial conducted on children with refractory epilepsy, the aqueous extract of the black seed was administered as an adjunct therapy and the effects were compared with those of a placebo. It can be concluded that the water extract of *Nigella sativa* seeds has antiepileptic effects in children with refractory seizures (Akhondian *et al.* 2007). In folk medicine, black cumin (*Nigella sativa* L.) seeds have traditionally been used for a variety of applications including treatments related to respiratory health, stomach and intestinal health, kidney and liver function, circulatory and immune system support, and for the general overall well being of human kind (Anwar 2005). The *Nigella*

sativa seed has the ability to reduce lipid profile which is a major risk factor for coronary artery disease in cardiac patients (Tasawar *et al.* 2011).

Phytochemicals in *Nigella sativa* L. seed

Nigella sativa L. seed is a complex substance of phytochemicals, some of which have not yet been isolated, as they are highly unstable. The quinonic phenol compounds present in the seeds are volatile, heat labile and photo reactive. Many phytochemicals have been characterized, but the major ones are thymoquinone (27.8–57.0%), ρ-cymene (7.1–15.5%), carvacrol (5.8–11.6%), t-anethole (0.25–2.3%), 4-terpineol (2.0–6.6%) and longifoline (1.0–8.0%). Thymoquinone readily dimerizes to form dithymoquinone (El-Dakhakhny 1963). Four alkaloids have been reported as the constituents of the seeds, of these nigellicine (Atta-ur-Rahman *et al*. 1985a) and nigellidine (Atta-ur-Rahman *et al.* 1995) have an indazole nucleus, whereas nigellimine (Atta-ur-Rahman *et al.* 1992) and its N-oxide (Atta-ur-Rahman *et al.* 1985b) are isoquinolines. A monodesmosidic triterpene saponin, α-hederin, was isolated from the seeds, which was reported previously in *Hedera helix* leaves (Swamy and Benny 2001).

Investigations on the lipid compositions of *Nigella* species showed a total oil content ranging from 28.0 to 36.4%, having a major content of linoleic acid (40.3–58.9%) followed by oleic (18.7–28.1%), palmitic (10.1–12.5%), 22:1 (3.2–3.8%) and stearic (2.6–3.1%) acids (Matthaus and Ozcan 2011). All the *Nigella sativa* seed oils exhibited differences in their tocopherol content. The oil extracted from the seeds contained α- tocopherol (1.70–4.12mg/100g), β-tocopherol (4.90–17.91mg/100g) and γ- tocopherol (0.97–4.51mg/100g). The total tocopherol content in seeds varied between 9.15 and 24.65mg/100g of seed material. The total amounts of sterols ranged

from 1993.07 mg/kg to 2182.17 mg/kg. The main component was β-sitosterol (48.35–51.92%), followed by 5-avenasterol, campesterol, and stigmasterol.

A combination of volatile oils, fatty acids and trace elements are believed to contribute to the seeds' effectiveness (Salem 2005). The *Nigella sativa* seeds contain fixed oils, proteins, alkaloids, saponin (36–38%) and essential oil (0.4–2.5%). The fixed oil is composed mainly of unsaturated fatty acids, including the arachidic and eicosadienoic acids (Houghton *et al*., 1995; Burits and Bucar 2000).

Major quinone compound: Thymoquinone and its bioactivities

The *Nigella sativa* seed oil constituents a major quinone i.e thymoquinone (TQ) exhibited potential medicinal properties and potent anti-inflammatory effects (Mutabagani and El Mehdy 1997) on several inflammation-based models, including experimental encephalomyelitis, peritonitis, colitis, oedama and arthritis through the suppression of the inflammatory mediators prostaglandins and leukotriens (Chakrabarty *et al.* 2003). The oil and the active ingredient TQ showed beneficial immunomodulatory properties, augmenting the T cell and natural killer cell-mediated immune responses (Haq *et al.* 1999). Most importantly, both the oil and its active ingredient TQ exhibits anti-tumor and anti-cancer properties (Badary *et al.* 1999; Worthen *et al.* 1998). TQ in nanoparticles were more potent than TQ intact in suppressing proliferation of colon cancer, breast cancer, prostate cancer and multiple myeloma cells and also an encapsulation of TQ enhance its anti-proliferative, anti-inflammatory and chemosensitizing effects (Ravindran *et al.* 2010). As a most abundant component of black cumin, TQ has been reported to exhibit chemosensitization and chemopreventive potentials (Badary *et al.* 1999; Badary and Gamal El Din 2001; Gali Muhtasib *et al.* 2004).

Beneficial effects of *Nigella sativa* and TQ on histopathological changes of sciatic nerves in streptozotocin (STZ) induced diabetic rats were shown to cause a sharp decrease in the elevated serum glucose and an increase in the lowered serum insulin concentrations. No histopathological changes of sciatic nerves in STZ induced diabetic rats by black cumin and TQ treatment has also been reported (Kanter 2008).

TQ has been found to attenuate lipid peroxidation (Nagi *et al.* 1999) and ifosfamide Fanconi syndrome (Badary 1999). Pure TQ inhibits cyclooxygenase and 5-lipoxygenase pathways of arachidonate metabolism in rat peritoneal leukocytes with consequent inhibition of the formation of thromboxane B_2 and leucotriene B_4 (Houghton *et al.* 1995). Nigellone, the polymer of TQ inhibits histamine release from mast cells (Chakravarty 1993). In addition, it also protects kidney from cisplatin nephrotoxicity (Badary *et al.* 1997), liver from tetrachloride hepatotoxicity (Al Gharably *et al.* 1997) and heart from doxorubicin cardiotoxicity (Al Shabanah *et al.* 1998).

The inhibitory activity of TQ against the formation of advanced glycation end products was studied using the hemoglobin-δ-gluconolactone, human serum albumin-glucose and the *N*-acetyl-glycyl-lysine methyl ester-ribose assays. A comparison was made with the inhibitory activity of aminoguanidine. The cytotoxicity of TQ was studied by the release of lactate dehydrogenase from platelets and the levels of plasma thiols. At 20 μM, TQ inhibited 39% of hemoglobin glycation, 82% of post-Amadori glycation products, reduced methylglyoxal-mediated human serum albumin glycation by 68%, inhibited 78% of late glycation end products. Aminoguanidine at 10 mM was less effective than TQ. The IC_{50} for TQ and aminoguanidine were 7.2 μM and 1.25

mM, respectively. TQ at 20-50 μM was not toxic to platelet lactate dehydrogenase and plasma thiols (Losso *et al.* 2011).

Bioactivity of black cumin depends on the TQ content, the major component of the essential oil, which is also present in the fixed oil. Black cumin contains 30 to 40% oil and 20 to 30% protein with antioxidants lignans such as saponin, melantin (Ali and Blunden 2003; Tulukcu 2011).

Products from *Nigella sativa* L.

Several products containing *Nigella sativa* oil or seed powder in the form of gel capsule, vapor rub, herbal powder, cream etc are being made and marketed today for treating various health problems. For example BARAKA DIABsol (black seed oil soft gel capsules) is marketed for diabetes patients to reduce their blood glucose level. Other therapeutic and cosmetic products such as BARAKA black seed cream (Herbal antiseptic cream), Black seed Herbal Tea with Mint are available in the market. Consuming one teaspoon of oil after meals three times a day and inhalation of vapor of boiling water with 1 teaspoon of oil have been reported as the direction of use for asthma and cough. Intake of 1 teaspoon of oil with a cup of yoghurt 2 times daily suppresses the diarrhea related problems (Ilaiyaraja and Khanum 2010).

Photodimerization of thymoquinone (TQ)

The major quinonic phenol compounds (Dictionary of organic compounds 1953) are highly photo reactive, thermolabile, volatile and unstable and transform often via photodimerization. In earlier studies, the major quinone constituent of the seed was thought to be Dithymoquinone (DTQ) and was shown to be formed via photodimerization of TQ as a consequence of exposure to sunlight during separation and extraction procedures (El Dakhakhny 1963). Storage conditions also make a

difference in the amounts of the quinone constituents of the *Nigella sativa* seed oil, especially if seed oil samples are exposed to heat and light (Ghosheh *et al.* 1999). The transformations of different compounds were depicted as Fig. 1.3.

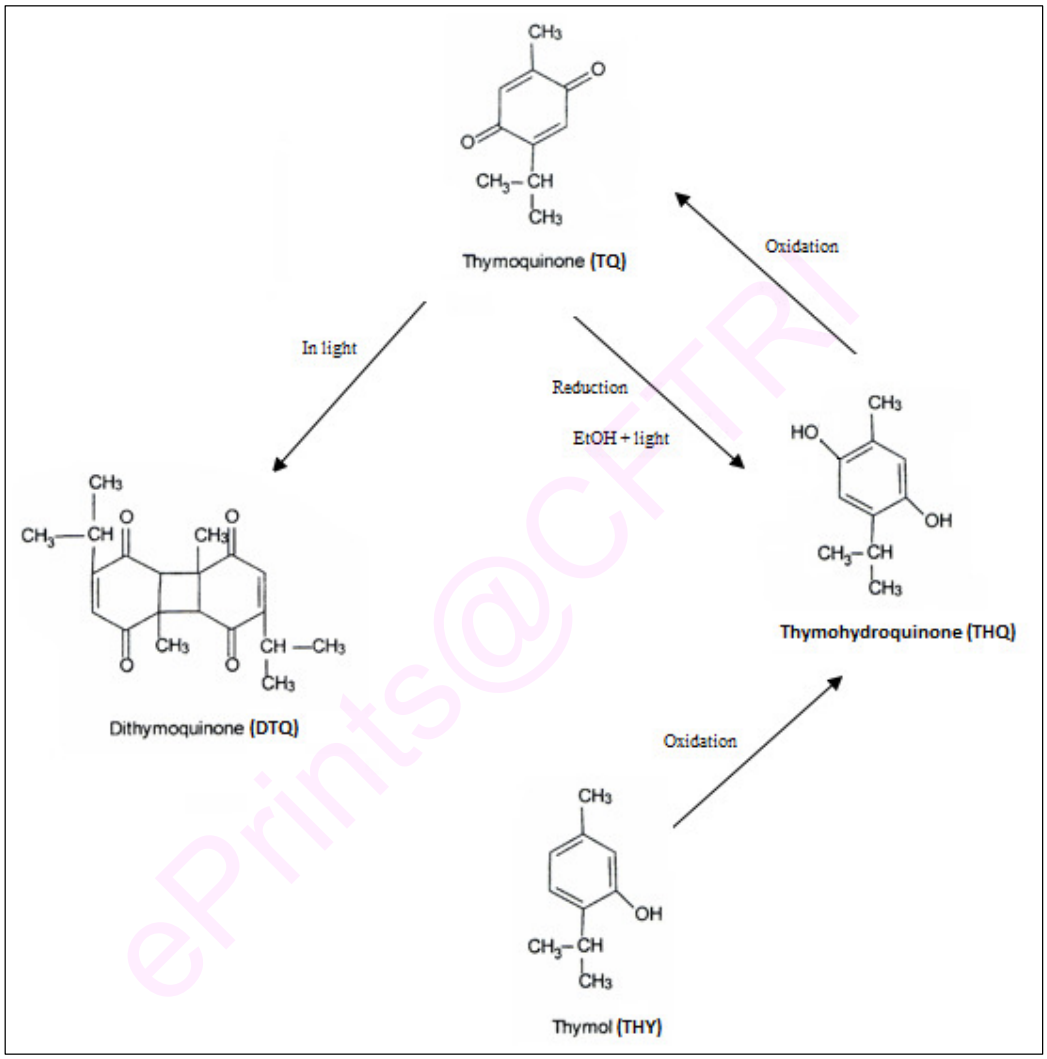


Fig. 1.3. Thymoquinone and thymol and their transformations

Extraction techniques

An extraction is usually the first step in analytical procedures, applied to the determination of organic compounds in solid matrices. From the extraction design point of view, issues like the source of the bioactive compounds; global or total yield obtained in the process; productivity and selectivity must be considered. These factors are directly linked to the economic viability of the selected industrial process and the quality and purity of the final product (Quispe-Condori *et al.* 2005). So far, several efficient extraction techniques have been developed and are commonly used for analyte isolation from solid matrices. There are two extraction procedures i.e conventional and supercritical fluid extraction (SFE), are currently utilized by food and pharmaceutical industries.

Conventional extraction

Most commonly used conventional extraction methods are steam distillation, soxhlet extraction (for volatiles) and Soxtec™ system for fixed oils and non volatile compounds. Very few investigators have reported conventional methods like hydro distillation and solvent extraction for extracting essential oils from seeds. In the *Nigella sativa* seed oil obtained through hydro-distillation process, the content of thymoquinone was only 3% against 48% obtained by soxhlet extraction (Burits and Bucar 2000). The *Nigella* seed oil extracted by using petroleum ether at 40–60°C revealed that the seed is a good source of oil and protein (Atta 2003).

Steam distillation

In Steam distillation method as heat energy of around 100°C is used to separate the low and medium molecular weight compounds causing an alteration of the thermo labile essential oil constituents. Also, the water/steam can exert a hydrolytic influence

that brings about chemical changes in the oil. This method consumes more energy and time for separating essential oils from plant products.

Solvent extraction

Solvent extraction is a principal mode for separating mixtures by using the differences in the solubility of the components. It is an efficient and complete mode for oil extraction, preferred by large cooking oil manufacturers. The oil-solvent mixture here is heated to about 150°C to evaporate out the solvent (e.g. Nuts and oil seeds) to obtain oil devoid of the solvent.

Soxtec™ system

This technique is an automated version of the classic Soxhlet approach to extracting solid samples. Soxtec™ is based on Soxhlet and Goldfisch extractions. This approach initially immerses the thimble containing the sample directly into the boiling solvent. Then, the thimble is moved above the solvent to mimic the rinse extraction step of Soxhlet extraction. Finally a concentration step using modern automated equipment reduces the final volume to 1-2ml. This three-stage approach shortens the extraction step to 2-3 hours, because it provides direct contact between the sample and solvent, at the solvents boiling point. After rinsing, almost 90% of used solvent is recovered.

Disadvantages of conventional extraction

Low yield, poor quality of extracts, loss of volatile compounds, thermo and oxidative degradation, toxic solvent residues, emission of VOC (Volatile organic compounds) and ODC (Ozone depleting compounds) to the environment, handling hazards of solvents, difficulty to remove the solvents from products, degradation of labile compounds resulting in off-flavour compounds and failure to extract unstable

organic compounds like phytochemicals. More over the conventional extraction procedures are highly expensive, requiring greater volume of organic solvents with longer extraction time.

Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) was developed in 1960 (Lin *et al*. 1999). In recent years, many studies have been reported the use of supercritical fluid extraction with carbon dioxide (CO_2) as a solvent for extraction of natural compounds from different raw materials. Fig 1.4 represents the molecular basis of SFE unit.

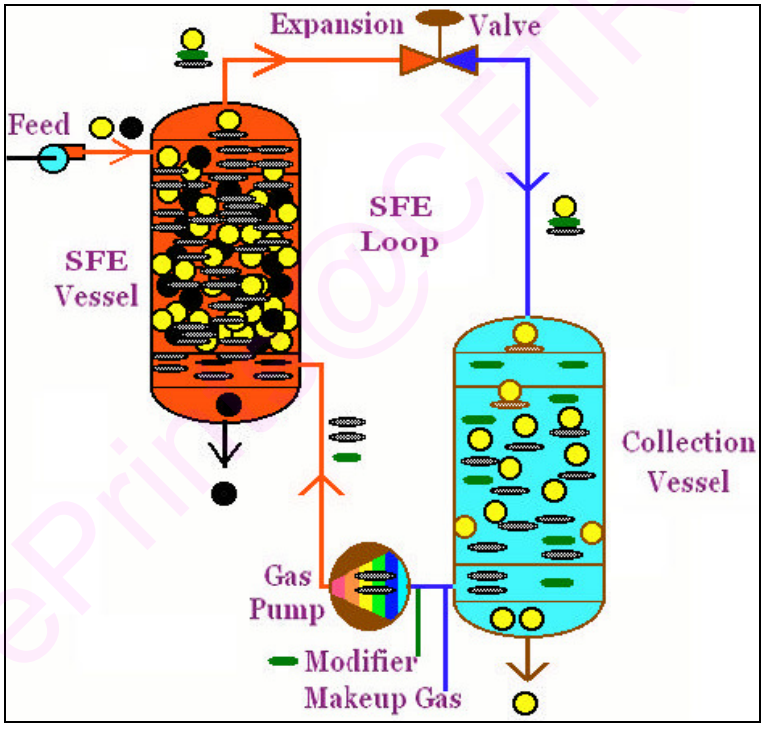


Fig. 1.4. Molecular basis of SFE unit

It is an attractive and alternative to conventional methods, due to the usage of environmentally compatible fluids, reduced solvent consumption, oxygen-free extraction environment, ease of separation of solute from supercritical fluid (SCF) solvent by simple expansion and shorter extraction time. The combined liquid-like

solvating capabilities and gas-like transporting properties of supercritical fluids make them particularly suitable for extracting bioactive compounds from plant tissues with a high degree of recovery in a short period of time. It was believed that, by using SFE, the extraction time can be reduced to tens of minutes compared with that by liquid–solid extraction that requires hours or days (Bimakr *et al*. 2011). SFE is an important process in the food, pharmaceutical and cosmetic industries because it is possible to develop products without toxic residues, with no degradation of active principles and with high purity. Studies on the extraction of essential oils, phenolic compounds, carotenoids, tocopherols, tocotrienols, alkaloids and other classes of chemical compounds have been published. Several matrices are used such as seeds, fruits, leaves, flowers, rhizomes, roots, peels of fruits, and branches of trees. One of the most important advantages of SFE is the possibility of changing and optimizing the operational conditions to facilitate the extraction of specific compounds. Selection of these conditions depends upon the specific compound or compound family to be extracted. Studies have revealed that extracts obtained by SFE maintain or exceed the bioactivity of extracts obtained by conventional techniques. The reason for this is the specificity of the SFE process, which promotes a selective extraction, resulting in an extract enriched in desirable compounds, free from solvents and without any loss of compounds due to degradation or reaction (Pereira and Meireles 2010).

SFE technology is increasingly gaining importance over the conventional techniques for extraction of natural products (Nyam *et al.* 2009). The solvating power of Supercritical fluids (SCF) is directly related to pressure and temperature. Due to the absence of toxic residues in the final product, supercritical fluids are especially useful for extracting valuable bioactive compounds (such as flavors, colorants, and other

biomolecules) and for removing undesirable compounds (such as organic pollutants, toxins, and pesticides). Several solvents are used in SFE. In fact, any solvent can be used as a supercritical solvent; however, the technical viability (critical properties), toxicity, cost, and salvation power determine the best-suited solvent for a particular application. Nevertheless, other solvents such as propane, ethane, hexane, pentane and butane have been investigated as SCF solvents (Pereira and Meireles 2010). Recently, extraction of heat labile natural compounds like phytochemicals, by using CO_2 has been considered as an interesting application, because of low operating temperature and lack of contamination of the product by solvents. The use of supercritical fluid extraction with carbon dioxide ($SCCO_2$) as a solvent (Ibanez *et al.* 1999) has increasing interest for the recovery of unaltered organic healthy compounds best suited for application in the food and pharmaceutical industries. Although several gases can be used for SFE, CO_2 is preferred as the extraction medium. Not only is it non- toxic, tasteless, odorless and inert, but also its critical point, 73.8 bar and 31.2^{o}C, permits the SFE to be carried out at near ambient temperatures. Moreover, the solubility of many compounds in it at medium pressures is appreciable. SFEs are advantageously applied to increasing product performance to levels that cannot be achieved by traditional processing technologies. Employing SFE technology in place of solvent extractions appears to be too expensive but the health benefits derived out of using the solvent free SFE oils in pharmaceutical, medical and nutraceutical applications are highly valuable (Vanessa *et al.* 2009; Certik and Horenitzky 1999).

The efficient extraction of fats from plant samples for analysis and application level studies must be rapid to protect the loss of unsaturated fatty acids from the effects of the presence of oxygen, alkali and metal ions (Liu *et al.* 2009a; Lang *et al.*

1992) and the supercritical extraction achieves the same more effectively than the conventional solvent extractions. Recent years, supercritical fluid extraction (SFE) has been widely used in many fields, especially in natural products (Herrero *et al.* 2010). It has been used for developing an ever-expanding niche in the food and pharmaceutical industries, whether it is used as a solvent for extraction or analyses. Now days, SFE is a really needed advanced method which can provide faster, more reliable, cleaner and cheaper method for optimal extraction of natural compounds.

Applications of SFE

SFE have been used in different fields such as food, pharmaceutical, chemical, and fuel industries. The major application of SFE is in food and flavoring industry like the decaffeination of tea and coffee, the extraction of essential oils and aroma materials from spices and the extraction of *hops* in the brewery industry (Vollbrecht 1982). SFE processes are being commercialized in the polymer, pharmaceutical specialty lubricant and fine chemical industries. This method is used in extracting some edible oils from various seeds (Zhang *et al*. 2010) and also in the production of cholesterol-free egg powder. SFE has also proved effective in the separation of essential oils and its derivatives for use in the food, cosmetics and pharmaceutical industries, producing high-quality essential oils with commercially more satisfactory compositions (lower monoterpenes) than obtained with conventional extraction procedures including hydro-distillation (Ehlers *et al.* 2001; Diaz Maroto *et al.* 2002; Ozer *et al.* 1996). Other applications of SFE in food and flavoring industries are like extractions of tobacco (Hubert and Vitzthum 1978), color from paprika (Coenen and Hagen 1983), vanilla (Vidal *et al.* 1989), oil from oil seeds and oil bearing materials (Liu *et al.* 2009a; Cahoon *et al.* 2007; Eggers *et al.* 1985; Bulley *et al.* 1984; Friedrich

and Pryde 1984), flavors and fragrance (Caragary 1981; Calame and Steiner 1982), lipids and cholesterol from meat and fish (Chao *et al.* 1991; Froning and Wehling 1990), fractionation of fish oil esters (Nilson *et al.* 1989), fruit juices and concentrated essence, black pepper essential oil (Vidal *et al.* 1989), jasmine absolute (Udaya Sankar 1991), mushroom oleoresins, antioxidants, de-oiling of crude lecithin (Stahl and Quirin 1985), de-acidification of olive oils etc. In pharmaceutical industries major application of SFE is for extraction of active ingredients (phytochemicals) from herbal plants for avoiding thermo/chemical degradation and elimination of residual solvents from the products. Other applications are production of de-nicotined tobacco, environmental protection, purification of contaminated soil etc (Vanessa *et al*. 2009; Certik and Horenitzky 1999).

Considerable research interest has been devoted worldwide to investigate the *Nigella sativa* seeds for their phytochemicals especially thymoquinone (TQ) for its pharmacological properties. Many of these activities have been attributed to quinone compounds of TQ, DTQ, THQ and THY in the seed (Ghosheh *et al.* 1999). Coupling these beneficial effects with its usage in folk medicine, *Nigella sativa* seed is a promising source for bioactive ingredients with potential therapeutic modalities for different clinical settings. Thus, *Nigella sativa* seed is a complex substance of more than 100 constituents, some of which have not yet been identified or studied (Salem 2005). Based on the literature survey, it is clear that only limited works have been carried out on comparative extraction (through the supercritical CO_2 and conventional extraction procedures) of *Nigella sativa* seeds for its phytochemicals especially thymoquinone and its characterization studies, including antibacterial and antioxidant properties. Therefore, in this present study *Nigella sativa* seeds have been proposed.

1.2. Objectives

In view of the above, the present research work focuses on extraction of bioactive compounds especially for the major compound thymoquinone from *Nigella sativa* L. seeds by conventional as well as supercritical carbon dioxide extraction modes for food and pharmacological applications. Hence, the following objectives were undertaken in this study.

- ► Qualitative and quantitative comparison of the extraction for bioactive molecules from black cumin (*Nigella sativa* L.,) using conventional solvent and supercritical fluid carbon dioxide extraction procedures.
- ► Isolation and characterization of bioactive molecules from black cumin extracts.
- ► Evaluation of antioxidant and antimicrobial properties of black cumin extracts.

1.3. Scope of the present investigation

It is evident from the earlier sections that the bioactive principles of *Nigella sativa* L. seed extracts are excellent phytochemicals with potential bioactive properties. The importance of $SCCO_2$ process in extracting such phytochemicals from natural resources offers a non destructive method especially, in case of phytochemicals with potent bioactive properties from their sources. Thus, the present study was undertaken with the aim of extracting bioactive principles from *Nigella sativa* L. seeds and characterizing their bioactive properties.

Extraction conditions by conventional Soxtec^{TM} and $SCCO_2$ were investigated in great detail for extraction and enrichment of bioactive principles. The major bioactive principles were isolated through conventional chromatographic techniques

and characterized by a detailed spectroscopic investigation employing UV, IR, Mass, GC-MS and NMR (one and two dimensional).

The present study focused on four major compounds of *Nigella sativa* L. seed extracts, namely, thymol, thymoquinone, thymohydroquinone and dithymoquinone. Antibacterial and antioxidant properties were evaluated for the extracts and also for isolated compounds.

In order to derivatise these compounds for enhanced water solubility leading to better pharmacological properties, representative major compounds of the extract, namely, thymol was linked to carbohydrate molecules through enzymatic glycosylation. The synthesized thymol glycosides were also characterized spectroscopically.

Thus, this study has focused on extraction of *Nigella sativa* L. seeds, isolation and characterization of major bioactive principles and derivatisation of representative bioactive compound for better bioactive properties.

CHAPTER 2

Supercritical carbon dioxide ($SCCO_2$) and conventional solvent extractions of *Nigella sativa* L. seeds

CHAPTER 2

2.1. Introduction

The use of herbal medicines is as old as the existence of man on earth. From the very beginning, man knew that some plants were useful in curing certain ailments, while others were not and some were even harmful. The traditional knowledge was transferred from generation to generation. Nowadays, there is an increasing trend throughout the world to go back to nature, seeking what is safe and proved effective through clinical or long folk usage, while many conventional medicines are derived from plant extracts and some of them are still obtained directly from plants. Many pure compounds including alkaloids, glycosides and phenolics have already been discovered and others still continue to be discovered. Many herbalists believe that the therapeutic benefit of a medicinal plant resides in the whole herb as all its compounds work synergically, though there is little evidence to show that synergism occurs with most herbal preparations.

Growing side effects of synthetic drugs, demand for instant and processed foods, ever bulging costs of organic solvents and worrying health concerns related to them and awareness for environmental safety have led to the development of newer, sophisticated and quality separation/extraction techniques for food processing industries and pharma product ranges. Conventional separation techniques like leaching, adsorption, extraction and distillation, involve high temperature operations and use of toxic solvents leading to the deterioration and instability of various food constituents. Organic solvents are expensive, hazardous to use and costly to dispose of. Concern about the environment has increased the interest in alternative and reliable extraction techniques. To overcome this, efforts have been made to develop newer separation techniques like supercritical fluid extraction (SFE). Its non-toxicity

and non-flammability are at least two major reasons why carbon dioxide is the supercritical fluid in most common use. In addition, in the case of food applications, the lack of solvent residues is a huge advantage (Ollanketo *et al.* 2001). Some of the advantages of supercritical fluid extraction over conventional methods are high mass transfer rate, ease of product separation from residual solvent, selective product separation and better product quality. Supercritical fluid is a fluid at a temperature and pressure above its critical point. The critical point of a substance is one at which vapor phase and liquid phase become identical (single phase). A critical temperature is the temperature above which it is almost impossible to obtain two phases (liquid and vapor), whatever the pressure is applied and the pressure at that temperature is the critical pressure.

Supercritical fluids

A supercritical fluid is a state of any substance or compound at a temperature and pressure above its thermodynamic critical point (T_C and P_C). It has the unique ability to diffuse through matrix solids like a gas, and dissolve materials like a liquid. Additionally, it can readily change its density upon minor changes in temperature or pressure. These properties make it suitable as a substitute for organic solvents and carbon dioxide and water are the most commonly used supercritical fluids. Supercritical fluids (SCFs) are increasingly replacing the organic solvents that are used in industrial purification and recrystallization operations because of regulatory and environmental conservation pressures on hydrocarbon and ozone-depleting compounds (ODC). SCF-based processes have helped to eliminate the usage of hexane and methylene chloride as solvents. With increasing scrutiny of solvent residues in pharmaceuticals, medical products and nutraceuticals and with stricter regulations on VOC and ODC emissions, the use of SCFs is rapidly proliferating in

all industrial sectors. A supercritical fluid (SCF) is characterized by physical and thermal properties that are between those of the pure liquid and gas. The fluid density is a strong function of the temperature and pressure. The diffusivity of SCF is much higher than for a liquid and SCF readily penetrates porous and fibrous solids. Consequently, SCF can offer good catalytic activity.

Physico-chemical properties of Supercritical fluids

There are drastic changes in some important properties of a pure liquid as its temperature and pressure is increased approaching the thermodynamic critical point. For example, under thermodynamic equilibrium conditions, the visual distinction between liquid and gas phases, as well as the difference between the liquid and gas densities, disappear at and above the critical point. Similar drastic changes exist in properties of a liquid mixture as it approaches the thermodynamic critical loci of the mixture. Other properties of a liquid that change widely near the critical region are thermal conductivity, surface tension, constant-pressure, heat capacity and viscosity. Supercritical fluids (SCFs) have solvating powers similar to liquid organic solvents, but with higher diffusivities, lower viscosity and lower surface tension. Since the solvating power can be adjusted by changing the pressure or temperature, separation of analytes from solvent is fast and easy. These drastic changes make a supercritical fuel appreciably preferred over that of a liquid with the same density. By adding modifiers to a SCF (like methanol to CO_2) its polarity can be changed for having more selective separation power. SCFs are generally cheap, simple and many are safe. Disposal costs are also much less and in industrial processes, the fluids can be simple to recycle. SCF technology requires sensitive process control, which is a challenge. Further, it is expected that the combustion phenomena resulting from that of a SCF will be quite different from that of a liquid. In addition, the phase transition of the

mixture of solutes and solvents has to be measured or predicted quite accurately. Generally the phase transition in the critical region is rather complex and difficult to measure and predict.

A comparison of typical values for density, viscosity and diffusivity of gases, liquids, and SCFs is presented in Table 2.1. (Brunner 1994; Rizvi and Benado 1986).

Table 2.1. Comparison of physical properties of gases, liquids and Supercritical Fluids (SCFs).

Property	Gas	SCF	Liquid
Density (kg/m^3)	1	100–800	10^3
Viscosity (cP)	10^{-2}	0.05–0.1	0.5-1.0
Diffusivity (cm^2/s)	$10^{-2} - 10^{-1}$	10^{-4}–10^{-3}	10^{-5}

Applications of SCF include recovery of organics from oil shale, separations of biological fluids, bioseparation, petroleum recovery, crude de-asphalting and dewaxing, coal processing (reactive extraction and liquefaction), selective extraction of fragrances, oils and impurities from agricultural and food products, pollution control, combustion and many other applications.

Critical constants of supercritical fluids

Besides CO_2, the list of solvents which can be used as supercritical and sub critical fluids are given in Table 2.2. These fluids have been used for many applications such as in the extraction of paprika, carotenoids, tocopherols and capsaicinoids (Gnayfeed *et al.* 2001), and fluorinated hydrocarbons. But, till now, some of these solvents have not been used in the field of food and fragrance extraction. However, several other supercritical fluids (SCFs) have also been used in both commercial and development processes. Beside CO_2, water is another

increasingly applied solvent. One of the unique properties of water is that, above its critical point (374°C, 218 atm); it becomes an excellent solvent for organic compounds and a very poor solvent for inorganic salts. This property gives the chance for using the same solvent to extract the inorganic and the organic components respectively (Reid *et al*. 1987; Williams 1981; Mc Hugh and Krukonis 1986).

Table 2.2. Critical constants of some compounds used as Supercritical fluid

Compound	Critical temperature T_c (°C)	Critical pressure P_c (bar)
Benzene	289.00	48.30
$CClF_3$	29.00	38.00
Chlorotrifluoromethane	28.90	38.70
CO_2	31.10	73.00
Cyclohexane	280.30	40.20
Ethane	32.00	48.00
Ethylene	10.00	51.00
H_2O	374.00	218.00
Isopropanol	235.20	47.00
Methanol	240.00	78.00
N_2O	36.00	72.00
NH_3	132.00	112.00
p-Xylene	343.10	34.70
Propane	96.70	41.90
Propylene	91.90	45.60
Toluene	318.60	40.60
Trichloro fluoroethane	198.10	43.50

Advantages of CO_2 as a supercritical fluid

The choice of the SCF solvents is similar to the regular extraction and the principles of considerations are like good solving property, inert to the product, easy separation from the product and cheap costs. Carbon dioxide is the most commonly used SCF, primarily due to its low critical parameters (31.2°C, 73.8 bar), low cost and non-toxicity. Being non-toxic and physiologically harmless, the Supercritical carbon dioxide ($SCCO_2$) has GRAS (Generally Regarded As Safe) status. Also, the energy costs associated with this novel extraction process are lower than the costs for

traditional solvent extraction methods. Supercritical CO_2 extraction has been considered as a possible applied field of SFE, because CO_2 is nonflammable, the lack of a chemical residue problem and low critical temperature (31.2^oC) are important (Machmudah *et al.* 2005).

CO_2 is readily available and easy to remove from extracted products by simple solvent recovery. CO_2 is totally accepted for use with food products. Extraction with CO_2 usually takes place at low temperatures and in an inert atmosphere, whereby both thermal as well as oxidative degradations are prevented. Hence, selective extraction and fractionated separation is possible. Purer and high quality extracts with higher yield within shorter extraction period is possible with $SCCO_2$. CO_2 can be separated from the final product easily and completely because of its high volatility at low temperature. Solvent-free products covering all legal requirements are thus obtained and fragrances and aromas remain unchanged. The physical properties of CO_2 that make it widely used in the extraction process are low surface tension and viscosity and high diffusivity. The diffusivity of supercritical fluids is one to two orders of magnitude greater than those of other liquids, which permit rapid mass transfer, resulting in a larger extraction rate than that obtained by conventional methods (Ueno *et al.* 2008). Moreover, CO_2 is completely harmless and is even slightly germicidal. In addition, $SCCO_2$ is a solvent-free extraction and environmentally benign technology with the possibility of recovering intact natural compounds with minimal alteration of the active ingredients and preservation of the curative properties (Moyler and Heath 1988; Reverchon and Senatore 1992).

Principle of SFE

The basic principle of SFE extraction is that the solubility of a given compound/ solute in a SCF solvent varies with both temperature and pressure. At ambient conditions (25°C and 1 bar) the solubility of a solute in a gas is usually related directly to the vapor pressure of the solute and is generally negligible. In a SFE, however, solute solubility is up by 10-1000 orders of magnitude greater than those predicted by ideal gas law behavior. In Fig. 2.1, the critical point is marked at the end of the gas-liquid equilibrium curve and the shaded area indicates the supercritical fluid region (Brunner 1994).

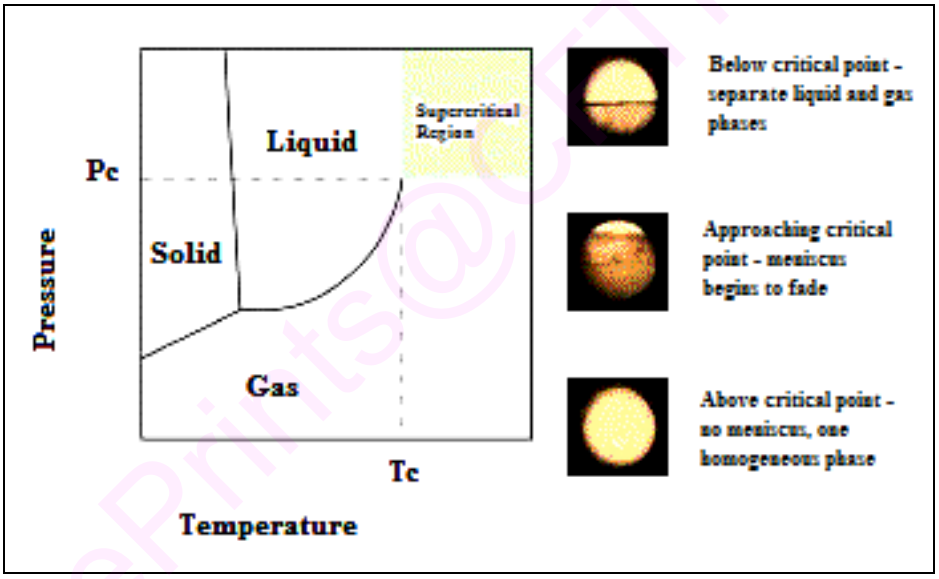


Fig. 2.1. Phase diagram and disappearance of meniscus between liquid and gas at the critical point.

It can be shown that by using a combination of isobaric changes in temperature with isothermal changes in pressure, it is possible to convert a pure component from a liquid to a gas (and vice versa) via the supercritical region without incurring a phase transition. Among various supercritical fluids, carbon dioxide as a supercritical solvent plays a key role in food, pharmaceutical and nutraceutical applications because of its inherent advantages. Carbon dioxide below its critical point (31.05°C,

7.38 MPa) exists as a liquid vapor and above its critical point it exists as a supercritical fluid (Udaya Sankar 1991). The boiling point and heat of evaporation are low and hence it is easy to separate from the solute with a lesser expense of heat energies. The selectivity of extraction is due to a wide variation of density with a slight variation in temperature. As carbon dioxide is inert, the extraction takes place in an inert atmosphere devoid of the problem of residual solvent in the final product.

Process description

The SFE extraction process essentially involves two steps, a loading step, in which the SCF solvent is brought into contact with the natural substance and a separation step, in which the material is separated by varying the physico-chemical properties of the solvent: this involves decreasing the temperature or pressure, or both; or by increasing the temperature alone; or even by entraining in a suitable adsorbent. The density can be varied by decreasing the temperature and pressure below the critical point; by increasing temperature, while keeping the pressure constant; increasing the temperature alone; and or by increasing the pressure, so that there will be two separate liquid phases or gaseous phases. Thus, by varying the physico-chemical properties of the supercritical fluid, the material can be re-precipitated. Fractionation of the extract may also be possible. In Fig. 2.2, the variation of the solubility with pressure is used to separate the solute from the solvent. The solute-laden solvent is decompressed through an expansion valve, where the solubility decreases due to decrease in pressure, and the material separates out in the separator. The solute- free solvent is recompressed and reused for extraction (Rizvi and Benado 1986).

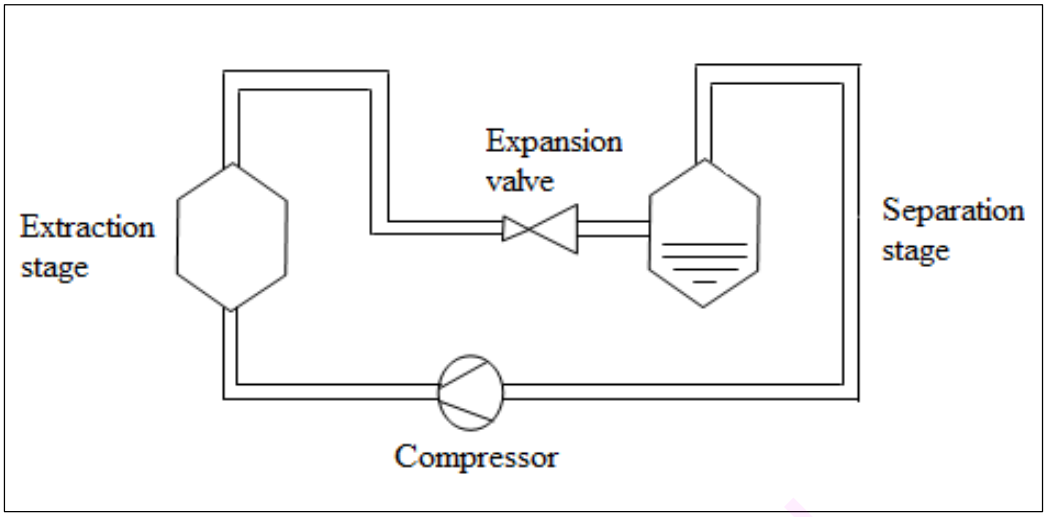


Fig. 2.2. Schematic process for supercritical fluid extraction utilizing pressure swing for separation of solvent and solute.

In Fig. 2.3, the temperature is varied to remove the desired extract from the SCF solvent. The extraction takes place at a temperature at which the solubility is at maximum, and the solute-laden solvent is then passed through a heat exchanger, where the temperature is so adjusted that the solubility of the component is at a minimum (Rizvi and Benado 1986).

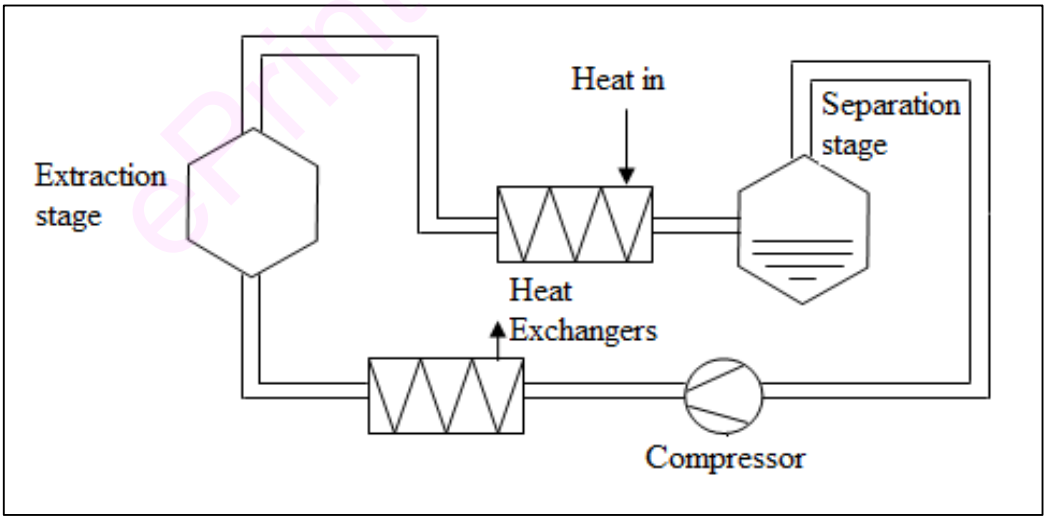


Fig. 2.3. Schematic process for supercritical fluid extraction utilizing temperature swing for separation of solvent and solute.

This allows the separation of the solute in the separator. The solute-free solvent is recompressed and reused.

$SCCO_2$ Extraction

The combined effects of high hydrostatic pressure and low acidity of an SCF can be beneficially employed in water-containing systems to prevent food spoilage by destroying bacteria (Kamihira *et al.* 1987). Rapid decompression of dissolved gas is sometimes used to expand and disrupt the cell structure of natural materials; hence, this technique could also be used as a means of sterilization. Although $SCCO_2$ can be an effective polar medium for enzymatic reactions (Taniguchi *et al.* 1987a; Van Eijs *et al.* 1988), it has also been used selectively to inactivate enzymes (Steytler *et al.* 1991; Taniguchi *et al.* 1987b; Weber 1980). In practice, this technique could be applied in situ, during an extraction process, or as a separate unit operation. Supercritical carbon dioxide ($SCCO_2$) is the most commonly used fluid for SFE of a variety of separations in food, bio-processing and analytical applications, because of its non-explosive, safety and low cost properties. However, SFE processes have been developed and standardized for varying plant materials (Zhang *et al.* 2010; Liu *et al.* 2009; Manninen *et al.* 1997; Cuperus *et al.* 1996; Favati *et al*. 1991) animal tissues (Devineni *et al*. 1997) and fungal cells (Sakaki *et al*. 1990).

Phase diagram for supercritical CO_2

In the pressure-temperature phase diagram (Fig. 2.4) the boiling line is observed, which separates the vapor and liquid region and ends in the critical point. At the critical point, the densities of the equilibrium liquid phase and the saturated vapor phases become equal, resulting in the formation of a single supercritical phase. The critical point is located at 304.1°K and 7.38 MPa (73.8 bar). With increasing temperatures, the liquid-vapor density gap decreases, up to the critical temperature, at which the discontinuity disappears. Thus, above the critical temperature a gas cannot

be liquefied by pressure. However, at extremely high pressures the fluid can solidify (Bott 1982).

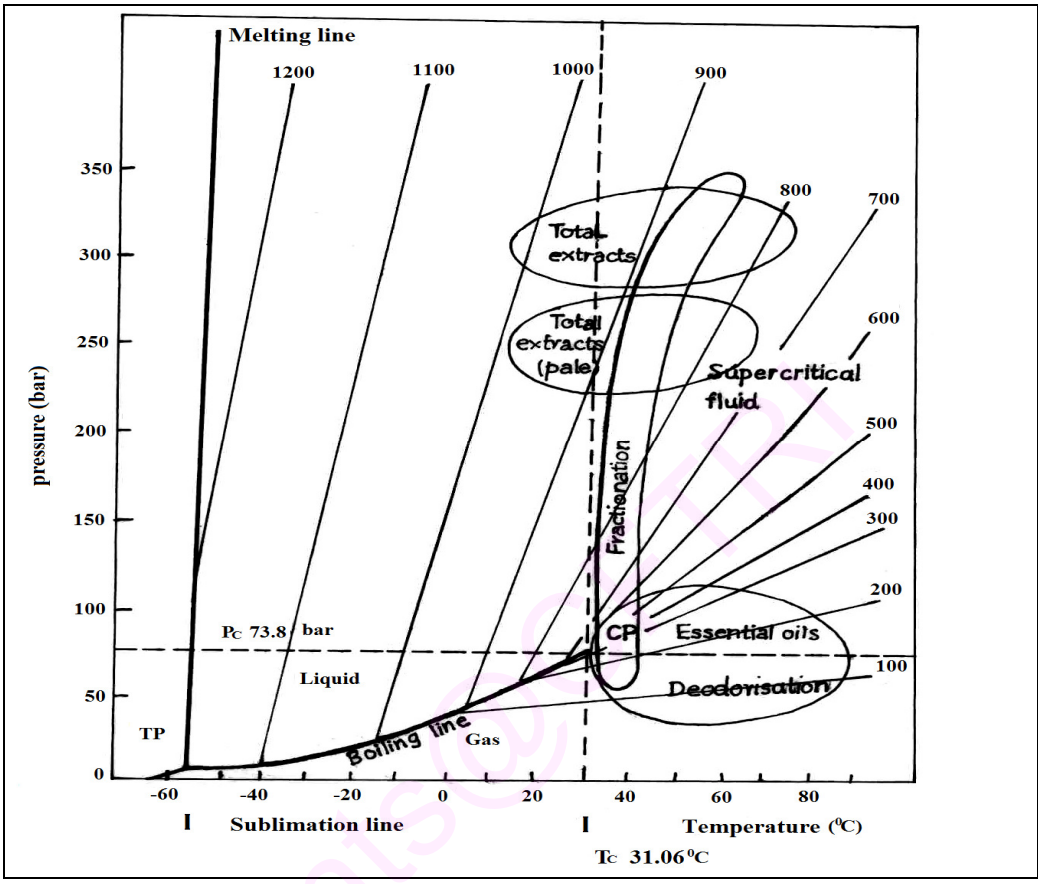


Fig. 2.4. Phase diagram of carbon dioxide demarcated for processing.

Many other physical properties also show larger gradients with pressure near the critical point, e.g. viscosity, the relative permittivity and the solvent strength, which are all closely related to the density. At higher temperatures, the fluid starts to behave like a gas, (Fig. 2.5) and for carbon dioxide at 400°K, the density increases almost linearly with pressure (Brunner 1994). Since the solubility of the components are usually reduced as the temperature decreases, most extractions with liquid CO_2 are carried out in the temperature range of −20°C to +20°C. However, when liquid CO_2 is employed at a higher temperature range (25°C to 31°C), the rate of extraction generally slows down as the critical temperature is approached. For a typical binary

solid-fluid system, the solubility isotherms exhibit a remarkable pressure-dependent behavior (Bott 1982).

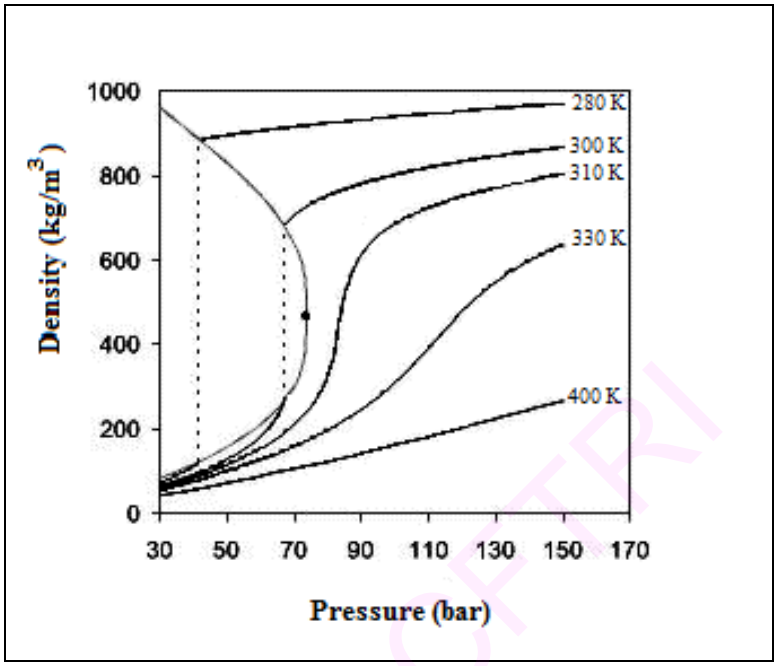


Fig. 2.5. Carbon dioxide density-pressure phase diagram.

It can be seen that the solubility of a solid solute initially decreases, reaches a minimum, and then exponentially increases with pressure in the vicinity of the critical pressure in the SCF state. So, its use as an extractant does not cause any further increase in the amount of CO_2 present in the earth's atmosphere. Therefore, there is no additional 'green house effect' from using CO_2 as the SCF solvent (Brunner 1994).

Response surface methodology (RSM)

RSM is a faster and more economical method for gathering research results than classic one-variable-at-a-time or full-factor experimentation (Liu *et al.* 2009b). RSM has been widely used in various research areas like food, chemical and biological processes (Linder *et al.* 1995; Bandaru *et al.* 2006; Ratnam *et al.* 2005; Ambati and Ayyanna 2001; Chen *et al.* 1997). This technique is used as an experimental study of relationship between response variables and significant input variables. It is an

optimization approach commonly used in industrial process control and engineering to find levels of input variables that optimize a particular response (Dhungana *et al.* 2006; Kaur *et al.* 2009). SFE along with RSM can be used to evolve newer procedures for pharmaceutical and food grade applications. Optimization of the experimental conditions is a critical step in developing a successful $SCCO_2$ process, because of the effect of variables on the extraction efficiency (Bimakr *et al.* 2011).

As thymoquinone (TQ) is volatile, heat labile and photo reactive, it is readily converted into dithymoquinone (DTQ) via photodimerization, when exposed to heat and sunlight, during conventional extraction of *Nigella sativa* seeds. Various storage conditions are also expected to make a difference in the amounts and quality of the quinone constituents, especially the TQ of the oil (El Dakhakhny 1963).

In this present study, supercritical CO_2 extraction of *Nigella sativa* seeds employing Box-Behnken experimental design and RSM technique has been carried out to maximize the TQ extraction.. Box-Behnken experimental design consisting of three independent variables i.e pressure, temperature and `solvent to material ratio' at three levels were studied on the thymoquinone (TQ) extracted. Conventional solvent extraction techniques using Soxtec^{TM} system with different solvents were also performed for qualitative and quantitative comparison of TQ.

2.2. Materials and methods

Plant material

Nigella sativa seeds were obtained from Supreem Pharmaceuticals Mysore Pvt.Ltd., India. Cleaned seeds were pulverized into a fine powder (20 mesh) before extraction by using an IKA-Analytical Mill A10 (Janke & Kunkel GmbH & Co. KG, Germany) to enhance the extraction efficiency and the powder was stored at a room temperature 25°C.

Chemicals

Standard Thymoquinone was purchased from Sigma-Aldrich, USA. Analytical grade solvents were obtained from Merck Co, India. The solvents were distilled once before use. Food grade CO_2 cylinder (99.9% purity) was obtained from Kiran Corporation, Mysore, India.

Determination of moisture content

The moisture content of the *Nigella sativa* seed powder was determined by toluene distillation method (ASTA 1985). 10 g of seed powder was taken in a round bottom flask along with 200 ml of toluene. Boiling chips were added to the flask to avoid bumping. The flask was connected to the condenser through a graduated Bidwell Sterling trap. A loose non absorbing cotton plug was inserted into the top of the condenser to prevent atmospheric moisture condensation. An electric heating mantle was used to reflux the seed powder for 4-5 hours. The amount of collected water was noted from the graduated trap and was used for calculating the percentage of moisture present in the seed. All the estimations were done in triplicates.

The moisture content was determined by using the formula

$$Moisture\,(\%) = \frac{Volume\ of\ water\ (mL)}{Weight\ of\ sample\ (g)} \times 100$$

Where *volume of water (mL)* means trapped water volume (in mL) in Bidwell-Sterling trap (5 mL capacity graduated in 0.1 mL. Intervals) and *weight of sample (g)* means used seed powder weight in grams.

Extraction phase

Extraction by Soxtec™ system

About 5 gm of black cumin seed powder was extracted separately in 120 ml of hexane, ethyl acetate, methanol and methanol:water (70:30% v/v) by using Soxtec™ system (Model: Soxtec™ system HT2 1045 Extraction unit, Foss Tecator, Sweden). The service unit temperature was adjusted to respective solvent's boiling temperature. Initially the sample loaded thimbles were placed in the extraction unit and were moved down for dipping in the solvent containing extraction cups for and then boiled at a temperature not exceeding the boiling point of the respective solvents for a period of 30 minutes and after that the thimbles were moved upwards for rinsing or extracting purpose with the solvent for a period of 3 hours. After rinsing, the solvent was recovered from the condenser (almost 90% of used solvent was recovered) and the extraction cups and the thimbles were removed. The collected extract was carefully taken from the extraction cups and made up to 50 ml in volumetric flask with respective solvent. 5 ml of extracts were taken in a preweighed petri dish as duplicates and allowed to dry for getting constant weight under vacuum to determine the extract yield (%). The remaining extracts were concentrated by evaporation of solvents using Buchi evaporator (model: RT111) under vacuum (40°C) to get extract (oil) which was collected along with ethanol and further subjected to stream of nitrogen. The residue/solvent free extract (oil) was collected and used for further studies.

Determination of Soxtec^{TM} extract yield

The extract yield on dry weight basis of seeds was determined by using the formula

$$Y = X \times \frac{(100 - M)}{100}$$

Where X is the weight in gms of the seed powder taken for extraction, M is the moisture content of the powder and Y is the weight of the seed powder on dry weight basis.

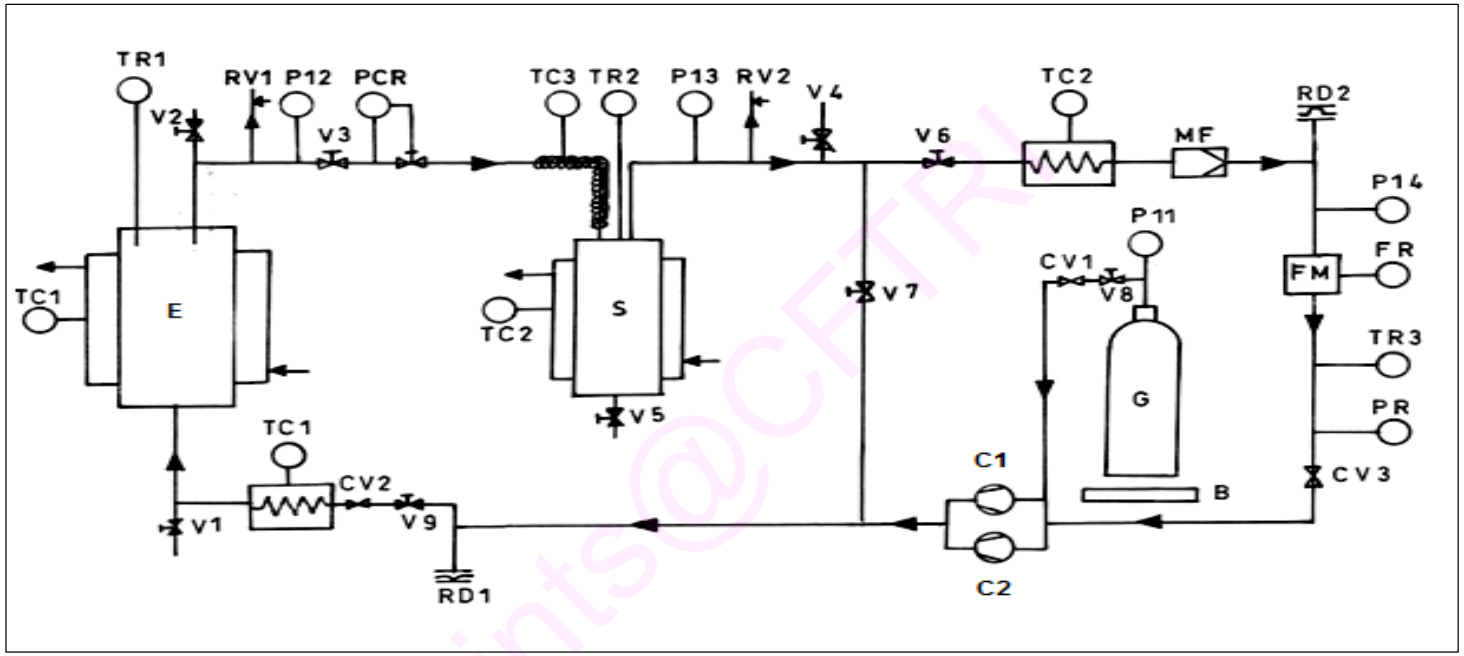
$$Extract\ yield\ (\%) = \frac{Weight\ of\ extract}{Y} \times 100$$

Extraction using Supercritical CO_2

A high pressure extractor (Nova Werke, AG, model Ex 1000-1.4-1.2 type, Switzerland) was used for the extractions (Figs. 2.6 and 2.7).

Fig. 2.6. Supercritical carbon dioxide extraction plant.

The extractor is capable of working to the maximum pressure of 1000 bar and at temperatures up to 100°C. Food grade CO_2 was pumped into the system by diaphragm pump until to set required pressure. Back pressure regulators were used to set the system pressure (in extractor and separator). The steel cylinder (1L) equipped with sintered metal plate on both ends was loaded with 1 kg of the dried and powdered material of black cumin seeds and then placed into the extractor vessel. Heat exchangers were provided in the system and on the extractor and separator vessel for temperature elevation. Before the CO_2 passed into the extraction vessel, it was pressurized to the desired pressure by the means of a diaphragm compressor and heated to the specified temperature by a heat exchanger to reach the supercritical state. $SCCO_2$ comes in contact with the material and flows through the extractor and enters the separator vessel through an expansion valve and was re-circulate after separation. A flow meter was provided to monitor the flow rate of CO_2 circulating in the system. Experiments were carried out at pressure of 80, 120 and 160 bar and temperature of 40, 50, and 60°C with `solvent to material ratio' of 15, 30 and 45 (kg/kg) at a CO_2 mass flow rate of 3.30-8.05 $\times 10^{-4}$ kg/s. After the extraction was completed, the extraction vessel was depressurized and the extract was collected by opening the valve located at the bottom of the separator vessel and was weighed. Further, the $SCCO_2$ extraction was carried out separately at 280 bar pressure and 50°C of temperature until no further extract was collected in the separator to make sure complete extraction was done. The total extract obtained was subjected to qualitative and quantitative comparisons.



TC 1-3	: Heat exchangers	FR	: Flow recorder
TR 1-3	: Temperature recorders	CV 1-3	: Check valve assemblies
V 1-9	: Valves	P 11-14	: Pressure indicators
PCR	: Pressure control regulator	C 1-2	: Compressors
RV 1-2	: Relief valve assemblies	B	: Balance
RD 1&2	: Rupture disc assemblies	PR	: Pressure recorder
MF	: Micro filter	E	: Extractor
FM	: Flow meter	S	: Separator

Fig. 2.7. Schematic diagram of supercritical carbon dioxide extractor

Determination of $SCCO_2$ extract yield

The extract yield on dry weight basis of seeds was obtained by using the formula

$$A = W \times \frac{(100 - M)}{100}$$

Where W is the weight of the loaded black cumin seed powder for $SCCO_2$ extraction, M is the moisture content of the seed powder and A is the weight of the seed powder on dry weight basis.

$$Extract\ yield\ (\%) = \frac{W_2 - W_1}{A} \times 100$$

Where W_2 is the extract weight along with plastic container, W_1 is the empty weight of that container. The weights are measured in grams.

Reverse-phase HPLC analysis

Quantification of TQ was performed on a HPLC system composed of a solvent delivery system (LC-10AT, Shimadzu Liquid Chromatography, Japan), column-oven, injector (CTO-10A, Shimadzu, Japan) and system controller (CBM-10A, Shimadzu, Japan) connected in a series to a photodiode array detector (SPD-M10 AVP, Shimadzu, Japan). Analysis was performed at room temperature on C18 reverse-phase μ Bondapak analytical column (300 x 3.9 mm i.d., 10 μm particle size, Waters Corporation, Manchester, UK). The isocratic mobile phase of water/methanol/2-propanol (50/45/5% v/v/v) at a flow rate of 1 mL/min was employed. The UV absorbance of the elutant was recorded at 254 nm. A calibration plot was constructed with the HPLC peak area of the injected pure thymoquinone (TQ) in the concentration range of 2—14μg of STD TQ in 1 ml of ethanol. A linear response with a correlation coefficient of 0.964 was obtained for the calibration curve (Fig. 2.8). About 1mg of every extract was dissolved in 1 ml of ethanol separately and 20μl of the respective

solutions were injected in to the HPLC column. TQ concentration of the every extract was determined from the calibration curve based from the peak areas of the TQ in the injected extract. Thymoquinone content in the samples (extracts) were expressed as % (w/w) on the basis of calibration curve of STD thymoquinone.

Determination of thymoquinone (TQ) extracted

Percentage of TQ extracted was determined using the following equation:

$$TQ\ Extracted\ (\%) = \left(\frac{X}{Z}\right)\times 100$$

Where *X* is the amount of TQ (g) in the extract and *Z* is the amount of the total TQ (g) in the loaded seed material.

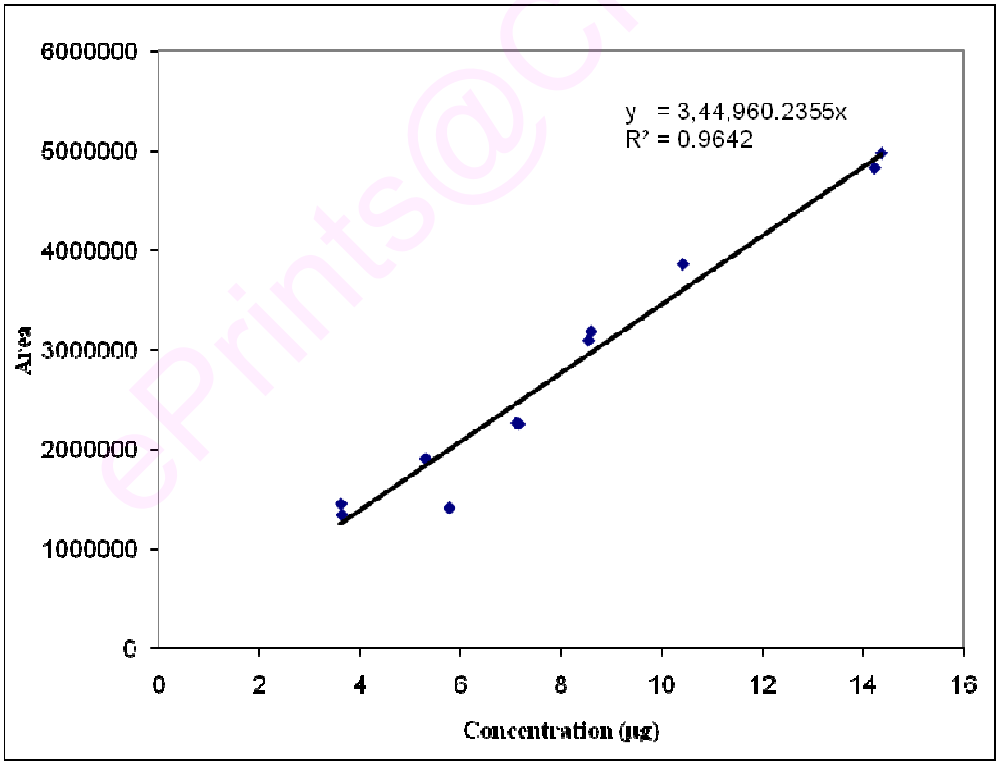


Fig. 2.8. Calibration curve for the standard thymoquinone (TQ).

Experimental design

Box-Behnken design (Box and Behnken 1960; Montgomery 1991) was employed for this RSM study. The experimental design which aided in investigating linear, quadratic and cross-product effects of three factors (like pressure, temperature and solvent to material ratio) each varied at three levels. The design involves 15 experiments from three variables at three levels of the system: pressure (80, 120 and 160 bar), temperature (40, 50 and 60°C) and solvent to material ratio (15, 30 and 45 kg/kg). RSM was applied to evaluate the effects of pressure (P), temperature (T) and solvent to material ratio (Sol:Mat) on extract yield and TQ extract. Lower extraction pressures of 80, 120 and 160 bar were chosen mainly to extract thymoquinone (TQ) preferentially, as the TQ is volatile compound and easily recovered by steam distillation. The density of carbon dioxide at the pressures chosen was most suitable for extraction of volatile oils (Udaya Sankar 1989). Further, the extract and its composition was a function of raw material and its composition (particle size, composition of extractives in terms of volatiles and non volatiles), pressure, temperature of carbon dioxide, time of extraction (solvent to material ratio). The composition of the extract in terms of volatile and non volatiles depends on the composition of the soluble extract in the raw material. Hence, TQ in the extract was a function of the available TQ in the raw material for extraction. Hence, extract yield and the % of TQ extracted in the extract were modeled in terms of a second order polynomial as a function of independent variables. The coded and uncoded independent variables used in the RSM design and their respective actual levels were listed in Table 2.3. A second order polynomial equation was used to express the extract yield and TQ extracted as a function of the independent variables.

$$Y = A_0 + A_1 X_1 + A_2 X_2 + A_3 X_3 + A_{11} X_1^2 + A_{22} X_2^2 + A_{33}$$

$$X_3^2 + A_{12} X_1 X_2 + A_{13} X_1 X_3 + A_{23} X_2 X_3 + \epsilon$$

Where, Y is the predicted extract yield or TQ extracted; X_1 is the pressure; X_2 is the temperature; X_3 is the solvent to material ratio; A_0 is the intercept; A_1, A_2 and A_3 are linear coefficients; ϵ is the error term. The yield of extract is expressed in percentage of total extract for 100gm of raw material, while the TQ extracted is also expressed as percentage of the TQ extracted in the extract to the total TQ in the raw material.

Table 2.3. Box-Behnken experimental design.

Expt. No.	Independent variables						Dependent variable	
	Coded			Actual			Extract yield (%)	TQ Extracted (%)
	Pressure	Temp.	Sol :Mat	Pressure (bar)	Temp. (°C)	Sol:Mat[#] (kg/kg)		
	X_1	X_2	X_3	X_1	X_2	X_3		
1	1	1	0	160	60	30	15.60	23.24
2	1	-1	0	160	40	30	30.20	34.88
3	-1	1	0	80	60	30	0.85	15.00
4	-1	-1	0	80	40	30	8.49	19.11
5	1	0	1	160	50	45	20.84	35.00
6	1	0	-1	160	50	15	16.93	22.49
7	-1	0	1	80	50	45	1.22	25.00
8	-1	0	-1	80	50	15	0.97	22.00
9	0	1	1	120	60	45	4.71	44.53
10	0	1	-1	120	60	15	2.42	38.48
11	0	-1	1	120	40	45	16.21	79.03
12	0	-1	-1	120	40	15	11.98	50.53
13	0	0	0	120	50	30	25.83	32.10
14	0	0	0	120	50	30	24.99	33.50
15	0	0	0	120	50	30	26.00	31.20

[#] Sol: Mat; solvent (CO_2) and material ratio.

The coefficients of the response function, their statistical significance and the process conditions for maximum extract yield and TQ extracted were evaluated by the least squares method using *KyPlot software* (KyPlot, Version 2.0 Beta). The response surfaces were also drawn using the KyPlot software, keeping one variable constant at mid-level and changing the other two variables within the range of experiments.

Statistical analysis

All the experiments were performed in triplicate and the data were calculated as means ± SD. A probability value of $P < 0.05$ was considered significant.

2.3. Results

Nigella sativa seed had a moisture content of 3%, determined by toluene distillation method and the extract yields were determined on the dry weight basis of the seed material.

Soxtec™ extracts

The Table 2.4 shows the yield of the total extracts calculated on dry weight basis of the *Nigella sativa* L. seeds. Various solvents can be used in the Soxtec™ extraction, but solvent selection depends upon the nature of compounds to be extracted from the seed material.

Table 2.4. Total extract yield in various solvents from *Nigella sativa* L. seed using Soxtec™ and $SCCO_2$ extractions

Extracts	Extract yield[#]
Soxtec™ extraction	
• Ethyl acetate	42.7 ± 1.21
• Methanol	37.3 ± 1.30
• Hexane	32.0 ± 1.01
• Methanol: water (70:30 v/v)	10.6 ± 0.48
$SCCO_2$ extraction	
• 280 bar, 50°C	33.0 ± 1.64

[#]Extract yields were expressed as % on dry weight basis of seeds;
The yields were an average for three independent experiments.

In Soxtec™ extraction, the highest extract yield was obtained in ethyl acetate (42.7%) and the lowest was in methanol: water (70:30 v/v) extract (10.6%). The highest extract yield in ethyl acetate might be due to the polarity of the solvent.

$SCCO_2$ extraction

Box-Behnken designed experiments were conducted and the results were analyzed by multiple regression analysis. The correlation between the experimental values and the predicted model values for the extract yields and TQ extracted are depicted in Figs. 2.9 and 2.10 respectively. The analysis of variance (ANOVA) for the fitted quadratic polynomial model is given for the extract yield as well as for the TQ extracted in the Table (Table 2.5). The regression coefficients of the fitted quadratic equation and standard errors for the extract yield and TQ extracted is given in Table 2.6. The study showed three linear coefficients (A_1, A_2 and A_3), three quadratic coefficients (A_{11}, A_{22}, and A_{33}) and three cross products (A_{12}, A_{13} and A_{23}). The design showed that polynomial regression models were in good agreement with the experimental results of extract yield as well as TQ extracted (Figs. 2.9 and 2.10; Tables 2.5 and 2.6). The model was built based on the variables with confidence levels of 95%. The ANOVA reported in Table 2.5 indicates that the model is highly significant, which is evident from the high Fisher (F) ratio value and low probability value. The coefficients of determination (R^2= 0.997) indicate a high degree of correlation between observed and predicted values of extract (Fig. 2.9) thus indicating an adequacy of the fitted model. The estimated coefficients of the regression model are depicted in Table 2.6. Data of the experimental yield were fitted on to a second-order polynomial. TQ Extracted and coefficients of determination (R^2= 0.945) indicate a good degree of correlation between the predicted and observed values of TQ (Fig. 2.10) which indicate a good fit of model. The ANOVA for TQ extracted is also presented in Table 2.5. The ANOVA represents that the model is highly significant at $P< 0.05$. It is established that the estimated coefficients of the regression

model for the experimental data of the TQ extracted fits to the second-order polynomial.

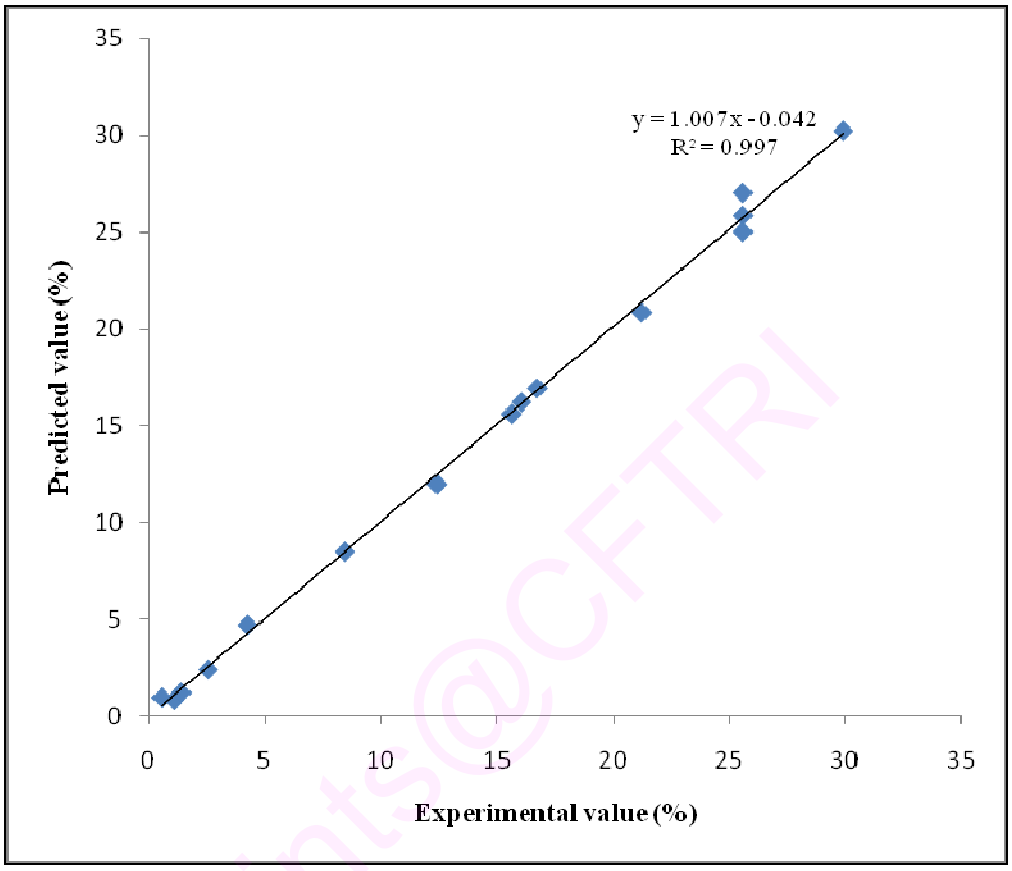


Fig. 2.9. Experimental values vs. predicted values for extract yields.

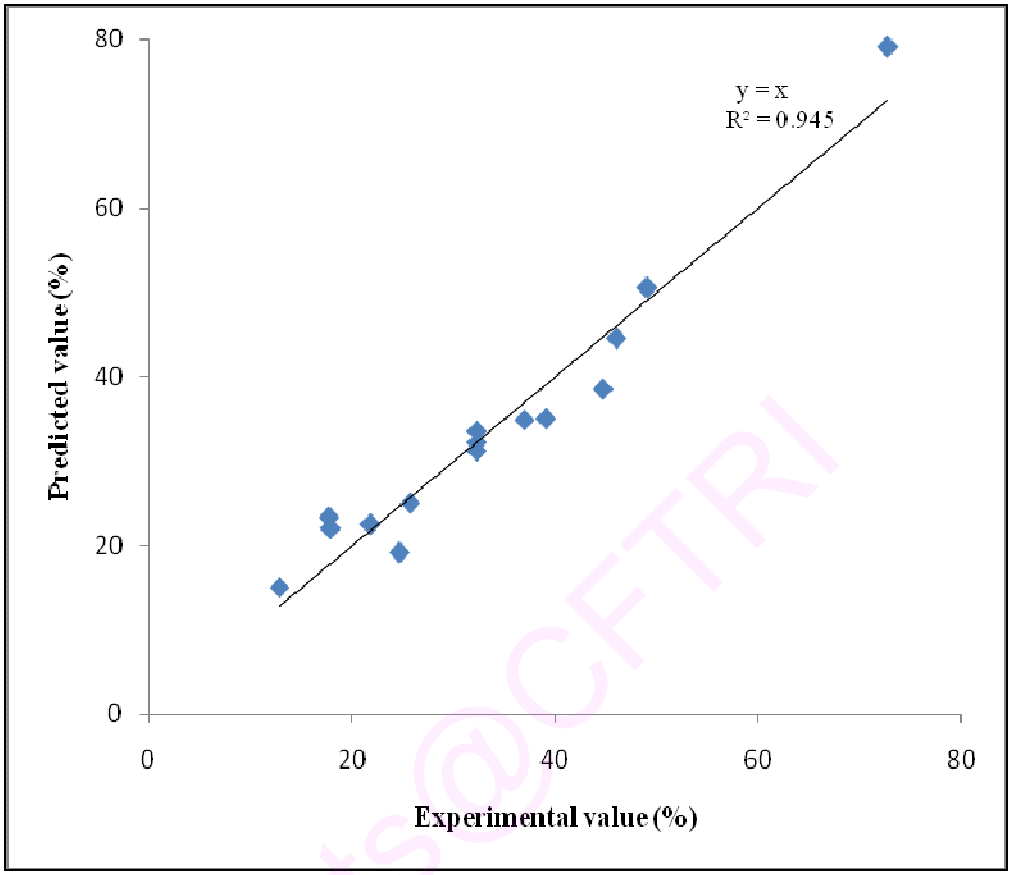


Fig. 2.10. Experimental values vs. predicted values for thymoquinone extracted.

Table 2.5. Analysis of variance (ANOVA) of fitted quadratic polynomial model.

		Degree of freedom	Sum of the square	Mean sum of the square	F ratio	Significance F	Regression Statistics
Extract yield	Regression	9	1488.797	165.421	532.954	6.50029E-07	R^2 =0.998
	Residual	5	1.551	0.310			Adjusted R^2 = 0.997
	Total	14	1490.349				Standard Error = 0.557
							n= 15
TQ Extracted	Regression	9	3310.887	367.876	9.710	0.011	R^2 =0.945
	Residual	5	189.430	37.886			Adjusted R^2 = 0.848
	Total	14	3500.318				Standard Error = 6.155
							n= 15

Table 2.6. Regression coefficients of the fitted quadratic equation and standard errors for the extract yield and TQ extracted.

	Extract			TQ		
	Coefficients	Standard error	*t* Stat	Coefficients	Standard error	*t* Stat
A_0	25.606	0.321	79.609	32.266	3.553	9.079*
A_1	9.005	0.196	45.717	4.312	2.176	1.981
A_2	-5.412	0.196	-27.478	-7.787	2.176	-3.578*
A_3	1.335	0.196	6.777*	6.257	2.176	2.875*
A_{11}	-5.330	0.289	-18.386	-18.114	3.203	-5.655*
A_{22}	-6.490	0.289	-22.387	8.905	3.203	2.780*
A_{33}	-10.285	0.289	-35.476	11.970	3.203	3.736*
A_{12}	-1.740	0.278	-6.246*	-1.882	3.077	-0.611
A_{13}	0.915	0.278	3.284*	2.377	3.077	0.772
A_{23}	-0.485	0.278	-1.741	-5.612	3.077	-1.823

*Significant at $p < 0.05$

Extract yield

Effects of pressure, temperature and solvent to material ratio

The surface response equation predicted a maximum of 29.94% (w/w) extract yield at a specific pressure (160 bar), temperature (40°C) to a solvent to material ratio (30 kg/kg) while the experimental yield observed was 30.20% (w/w) (Table 2.3). The response surface plots generated using the predicted equation showed the mutual interaction of extraction pressure, temperature and solvent to material ratio on the extract yield (Figs. 2.11, 2.12 and 2.13).

Interaction between pressure and temperature

The interactive effect of extraction pressure and temperature on extract yields were depicted in the Fig. 2.11. At lower temperature (< 50°C), increase in pressure has much favorable effect on the extract yields. On the other hand with substantial increase in temperature from 50 to 60°C the decrease in extraction yield was significant.

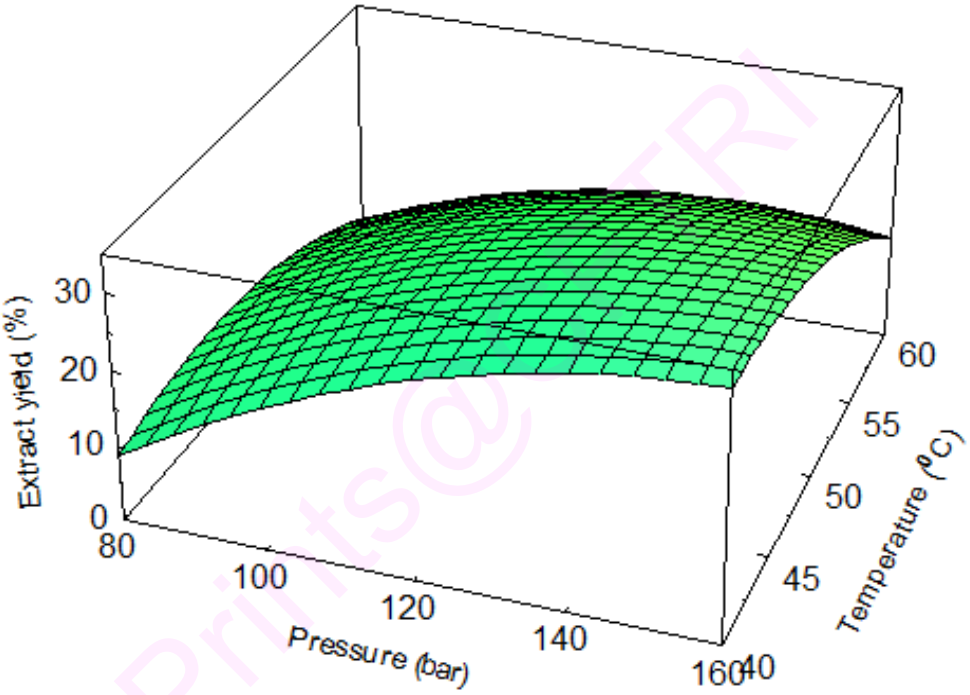


Fig. 2.11. Effect of the extraction pressure and temperature on the yield of *Nigella sativa* L. seed extract at solvent to material ratio of 30 kg/kg.

Thus, higher pressure (160 bar) along with substantial decrease in temperature (40°C) is responsible for quantitative recoveries and stronger interactions between the fluid and the matrix. High pressure (160 bar) and low temperature (40°C) conditions favor the higher extract yield whereas decrease in pressure from 160—80 bar and increase in temperature (from 40—60°C) did not favor the yield.

Interaction between pressure and solvent to material ratio

The Fig. 2.12 shows the interactive effect of pressure and solvent to material ratio on the extraction yield. It was observed that with an increase in pressure from 80 to 160 bar along with a substantial increase in the solvent to material ratio from 15 to 45 kg/kg, the extraction yields showed enhancements. Higher extract yield was obtained at higher concentrations of solvent to material ratio.

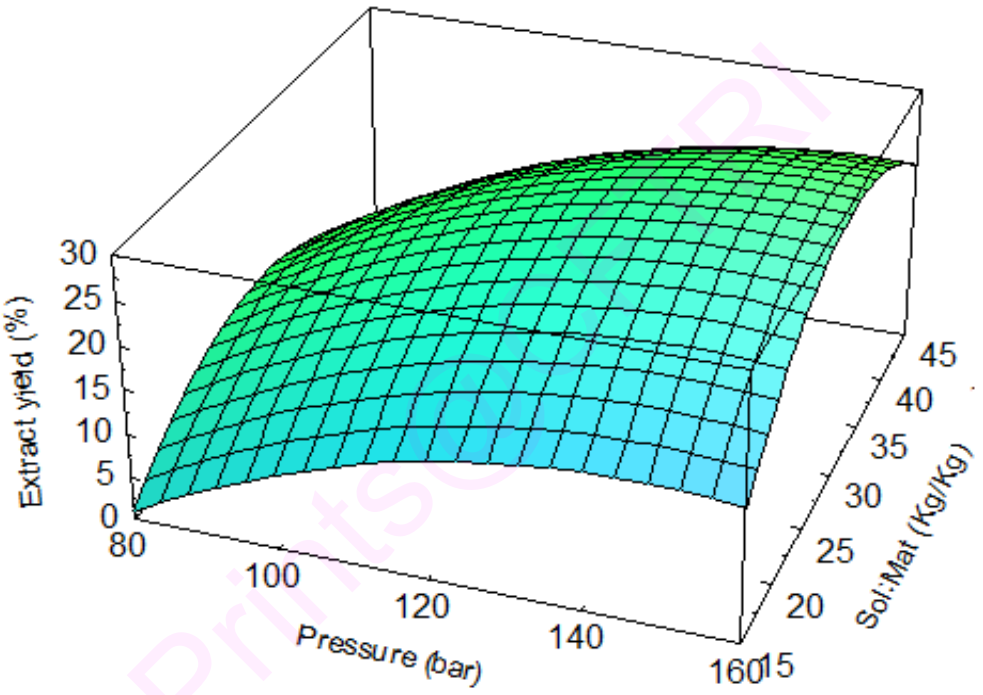


Fig. 2.12. Effect of the extraction pressure and solvent to material ratio on the yield of *Nigella sativa* L. seed extract at a temperature of 50°C.

Thus the extract yield gradually increased and reached a maximum at 30 (kg/kg) solvent to material ratio and a pressure of 160 bar of the tested range. High pressure increases the bulk density of the fluid mixture contributing to the solubility enhancement of the extract. For supercritical fluid extraction, the solubility of compounds depends largely on the balance between fluid density and solute vapor pressure, which are both controlled by fluid temperature and pressure. In other words,

the solubility of the solute may increase, remain constant, or decrease with increase in temperature at constant pressure, depending on whether the solute vapor pressure or the solvent density is the predominant factor.

Interaction between temperature and solvent to material ratio

The Fig. 2.13 shows the interactive effect of temperature and solvent to material ratio on the extract yield.

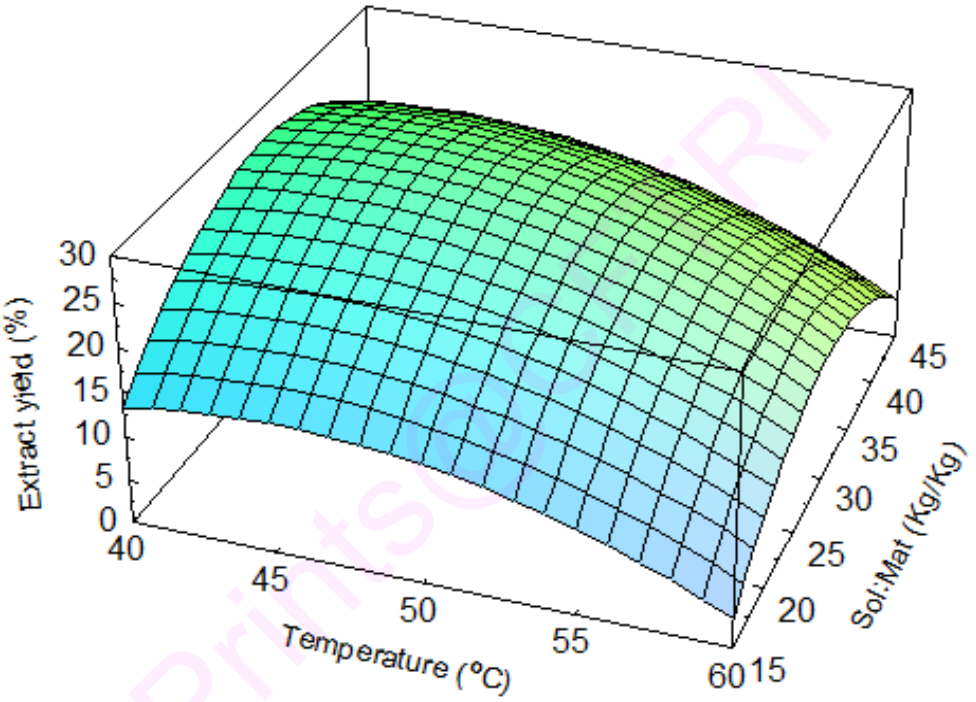


Fig. 2.13. Effect of the extraction temperature and solvent to material ratio on the yield of *Nigella sativa* L. seed extract at a pressure of 120 bar.

Thus the extraction yields gradually increased and reached a maximum at 30 kg/kg of solvent to material ratio and temperature between 45—50°C. An increasing solvent to material amount (up to 30 kg/kg) resulted in a higher extraction yield indicating there was a positive interaction between the temperature and mass of CO_2. It was also observed that with an increase in temperature (> 40°C) and solvent to material ratio (from 30—45 kg/kg), the extract yield decreased generally.

Thymoquinone (TQ) Extraction

Effects of pressure, temperature and solvent to material ratio

The maximum experimental amount of TQ extracted within the range of process conditions obtained was 79.03% (w/w) at a pressure of 120 bar, a temperature of 40°C and a solvent to material ratio of 45 kg/kg, while the predicted amount of TQ extracted was 72.80% (w/w). The mutual interaction of extraction pressure, temperature and solvent to material ratio on the TQ extracted are shown by response surface plots (Figs. 2.14, 2.15 and 2.16).

Interaction between pressure and temperature

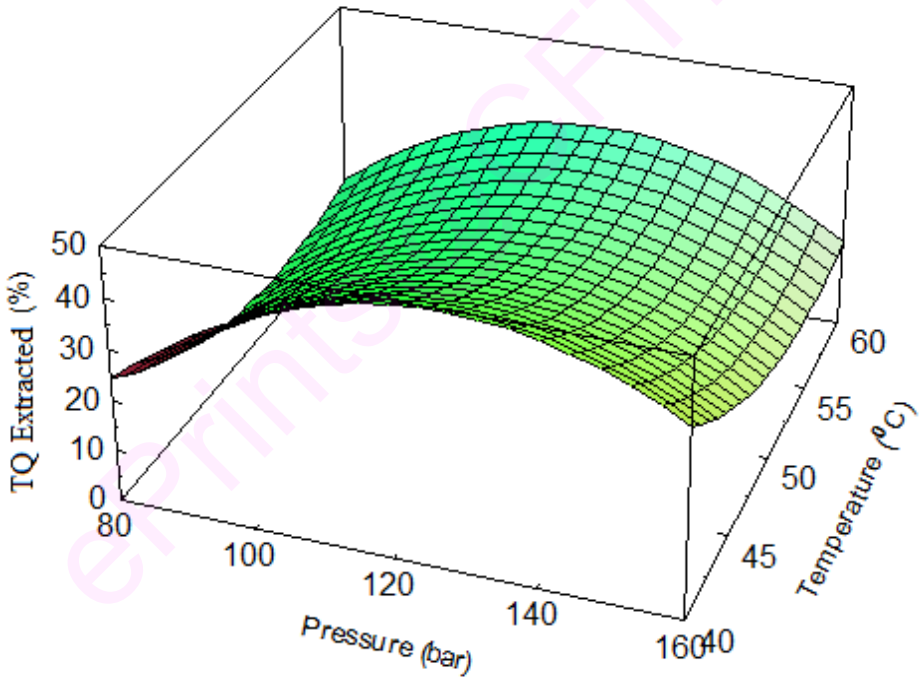


Fig. 2.14. Effect of the extraction pressure and temperature on TQ extracted from *Nigella sativa* L. seed at solvent to material ratio of 30 kg/kg.

The Fig. 2.14 shows that a temperature ranged between 40–60°C and an intermediate pressure (120 bar) conditions favored the TQ extracted in higher amount

whereas an increase/decrease of pressure from the point of 120 bar and also an increase in temperature didn't favor the TQ extracted in higher amount confirming the volatility and thermo sensitivity of TQ (El Dakhakhny 1963). Hence, the above results suggest that extraction of TQ by conventional extraction procedures involving higher temperature is not recommended.

Interaction between pressure and solvent to material ratio

It is observed that higher concentration of solvent to material ratio shows higher percentage of TQ extracted. TQ extraction was increased at 45 (kg/kg) of solvent to material ratio and a pressure of 120 bar of the tested range (Fig. 2.15).

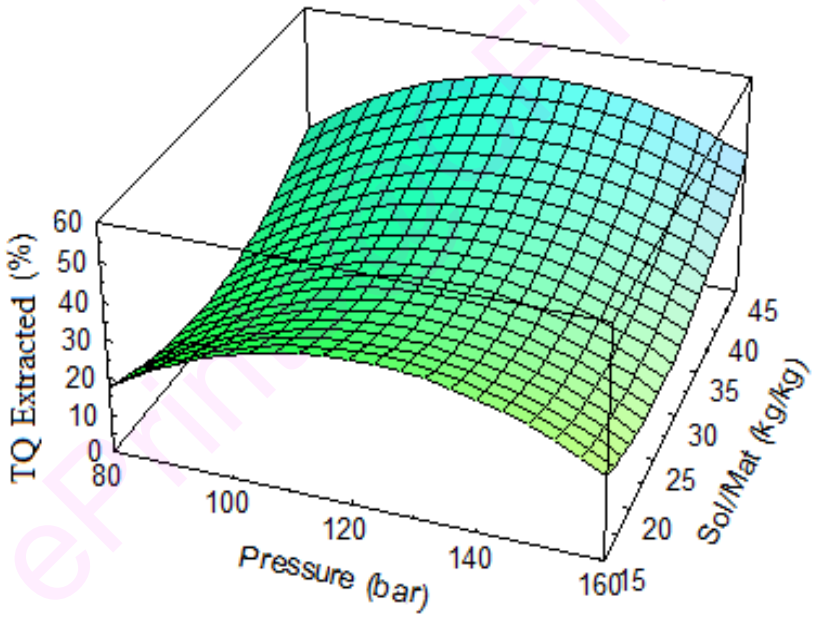


Fig. 2.15. Effect of the extraction pressure and solvent to material ratio on TQ extracted from *Nigella sativa* L. seed at a temperature of 50°C.

It indicates that there was a positive interaction between the pressure and the mass of CO_2 that could overcome the interface resistance for TQ which was transported from the seed matrix of *Nigella sativa* to CO_2 fluid.

Interaction between temperature and solvent to material ratio

It was observed that a decrease in temperature (60—40°C) and an increase in solvent to material ratio (15—45 kg/kg) generally favors higher amount of TQ extracted, whereas increase in temperature and decrease in solvent to material ratio did not favor the TQ extracted (Fig. 2.16). The composition of the seed extracts at different time intervals of extraction are studied with a view to find the TQ extracted. The early fractions showed a considerably lesser amount of TQ depending on the lower pressure and higher temperature of extraction. With an increased amount of solvent to material ratio up to 30 (kg/kg), the extract yield was increased logarithmically whereas TQ extraction was decreased and the latter fractions contain lesser amounts of TQ.

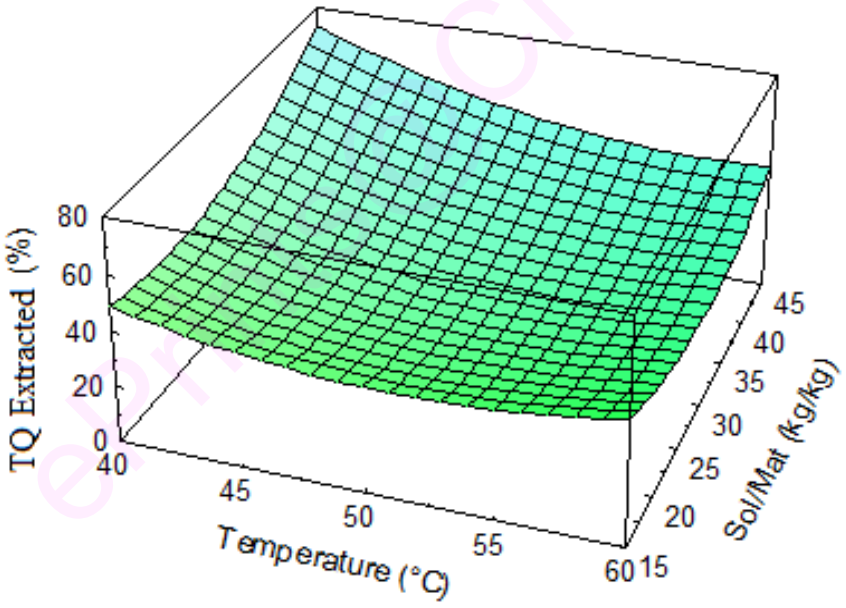


Fig. 2.16. Effect of the extraction temperature and solvent to material ratio on TQ extracted from *Nigella sativa* L. seed at a pressure of 120 bar.

The slope of the percentage of TQ extracted indicates that the TQ extracted is higher than the percentage yield of the extract. At higher solvent to material ratio, the TQ extracted was better. Extraction at a pressure of 80 bar and temperatures of 50 and 60°C, the TQ extracted was much less. It was observed clearly that there was a substantial amount of TQ extracted more than determined by the conventional methods. It is thereby clearly established that TQ extraction without any decomposition is possible only in $SCCO_2$.

Quantification of TQ

Nigella sativa seed various extracts were subjected to reverse phase HPLC analysis for TQ quantification and the results of total extracts are depicted in Table 2.7 and shows the TQ content in percentage as well as in raw material basis (%). TQ content in Soxtec™ extracts ranged from 0.6—3.2% whereas in raw material basis was ranged from 0.06—1.36%. The $SCCO_2$ extract (obtained as total extract at 280 bar pressure and 50°C of temperature) showed higher TQ content (7.1%) and TQ content in raw material basis was 2.34%. TQ content of the $SCCO_2$ extracts ranged from 2.5—12.3% depending on the process parameters of CO_2 extraction.

Among the extracts, the $SCCO_2$ extracts showed higher TQ content than conventional Soxtec™ extracts which showed lesser TQ content, as repeated extraction of the raw material in solvents during Soxtec™ extraction may probably transforming the TQ to DTQ or other quinonic phenol compounds, while in carbon dioxide extraction it is extracted without further transformation.

Table 2.7. Quantification of thymoquinone in total extracts of *Nigella sativa* L. seed obtained by Soxtec™ and $SCCO_2$ extractions.

Extracts	TQ content[a] (%)	TQ content in raw material basis (%)
Soxtec™ extraction		
❖ Ethyl acetate	3.2 ± 0.05	1.36
❖ Methanol	2.7 ± 0.16	1.00
❖ Hexane	1.2 ± 0.11	0.38
❖ Methanol: water (70:30 v/v)	0.6 ± 0.03	0.06
$SCCO_2$ extraction		
❖ 280 bar, 50°C	7.1 ± 0.32	2.34

[a]HPLC yields were determined as % of thymoquinone content; The yields were an average for three independent experiments.

Upon comparison, the conventional Soxtec™ extracts showed higher extract yields having lesser TQ content. Whereas the $SCCO_2$ extracts showed the lower extract yields but with the higher percentage of TQ extracted along with higher TQ content. The total extract obtained at 280 bar pressure and 50°C of temperature showed 7.1% as higher TQ content and 2.34% as higher TQ content in raw material basis, along with 33.0% as extract yield. From the above results it is clear that percentage of TQ extracted was higher in $SCCO_2$ extraction, indicating its superiority over other conventional extraction techniques.

2.4. Discussion

In this study, the increase in extraction yield in $SCCO_2$ fluid depends more on the solute's vapor effect. The results from the response plot showed that the most expressive factors that influence the extraction yield were temperature; interaction between temperature and SM ratio (solvent to material ratio) and pressure and SM ratio. These findings are in good agreement with our earlier reports (Suresh kumar *et al.* 2010a) where *Nigella sativa* seeds (extracted by $SCCO_2$ at 280 bar pressure and 50^{o}C temperature) showing higher extract yield than the extract obtained at 120 bar and 40^{o}C of temperature. In the present study, the maximum TQ content (of 12.3%) obtained at 120 bar, 40^{o}C and SM ratio of 45 kg/kg. It is thus proved that an intermediate pressure (120 bar) and temperature at 40°C conditions favored higher % of TQ extracted whereas an increase or decrease of pressure at the point of 120 bar and also an increase in temperature didn't result in higher % of TQ extracted. High pressure and temperature at 40°C and intermediate SM ratio of 30 kg/kg conditions favored higher extract yield but decreased TQ extraction. The extract yields of Soxtec™ extraction were higher but with lesser TQ contents compared to $SCCO_2$ extracts. It is very difficult to extract TQ, as it is highly reactive through the conventional Soxtec™ extraction which employs higher temperature and open place operating conditions. Kong *et al.* 2009 reported that an increase in temperature could accelerate the mass transfer of solute in the matrix and/or from the matrix to the solvent thus increasing the extraction yield. In general, temperature and solvent act as influencing factors, since solvents can penetrate regardless of the physical property of the sample matrix as affected by temperature making it easier for CO_2 to penetrate (Xu and Godber 2000). Similar concept was also documented in $SCCO_2$ extraction of a phytochemical: lycopene (Yi *et al.* 2009). In this study, $SCCO_2$ extract (120 bar,

40°C and SM ratio of 45 kg/kg) showed a maximum TQ content (of 12.3%) which correlates with our earlier reports (Suresh kumar *et al.* 2010a, 2010b). Reports on the proximate analysis of seed showed that it contained moisture 5.4%, lipids 37.3%, protein 20.0%, ash 6.7% and carbohydrate 30.5% (Khoddami *et al.* 2011).

In this study, the $SCCO_2$ fluid highly depends on the expressive factors of temperature; interaction between temperature and SM ratio; SM ratio and pressure solute's vapor effect which are influenced to get higher extract yield as well as higher % of TQ extracted from the seeds. It is indicated that an increase in pressure could accelerate the mass transfer of solute in the matrix and or from the matrix to the solvent thus increasing the extraction yield. Temperature and solvent acted as influencing factors as solvents can penetrate regardless of the physical property of the sample matrix as affected by the temperature making it easier for CO_2 to penetrate (Xu and Godber 2000). The favorable transport property of supercritical fluids near their critical points allows deeper penetration into solid seed matrix and hence effect more efficient and faster extractions than with conventional organic solvents. At high pressure and low temperature, the extraction yield increases as the degree of swelling in the seed matrix. The swollen matrix provides less mass transfer resistance and allows the intra particular movement of the extract to the particle surface. The swelling aids in the cell rupture thereby resulting in an increased surface area for exposing the active compounds in their intact forms, facilitating easy mass transfer into the CO_2.

2.5. Conclusion

In conclusion, the use of supercritical CO_2 in *Nigella sativa* seed extraction under specific optimal conditions resulted in a lower extract yield with a higher percentage of thymoquinone (TQ) extracted along with a higher TQ content when compared to solvent extractions. This optimization study confirmed the possibility of getting the TQ enriched extract through $SCCO_2$ extraction. The RSM study employed was for optimizing the operating parameters for increasing the percentage of TQ extracted or enrichment of TQ in black cumin seed extract. The RSM including experimental design and regression analysis was effective in developing an analysis model, with an optimal point for the factors affecting the $SCCO_2$ of extract from black cumin seed. This helps in assessing the effects of the factors (pressure, temperature, and solvent to material ratio) controlling the extraction of TQ from black cumin seeds. Hence, $SCCO_2$ was found suitable for black cumin seeds extraction in order to obtain an extract enriched with TQ. The experimentation carried out demonstrated that the $SCCO_2$ extraction can be used to obtain TQ enriched extract; the optimal extraction temperature was found to be 40°C while the pressure and solvent to material ratio were 120 bar and 45 kg/kg, respectively. The $SCCO_2$ technology has provided an impetus for the improvement in terms of quality as well as quantity of the extracted bioactive compound: TQ than any other conventional solvent extraction systems.

Followed by this, further characterization of *Nigella sativa* L. seed extracts were carried out as per the objectives of the research.

CHAPTER 3

Characterization of bioactive compounds

from

$SCCO_2$ extracts of *Nigella sativa* L. seeds

CHAPTER 3

3.1. Introduction

The interest in functional foods has increased dramatically in recent years as a result of the growing awareness of the health benefits of the ingestion of foods enriched with bioactive compounds, like phenolic compounds, carotenoids, tocopherols, tocotrienols, etc. These bioactive compounds belong to different classes of chemicals; hence they can be obtained by specific extraction techniques. A large number of medicinal plants and their purified constituents have been shown to have potential bioactive benefits. The rapid emergence of bioactive compounds as health products, has created a new trend for food processing and pharmaceutical industries.

The detailed knowledge about valuable bioactive profiles is mandatory for food and pharmaceutical applications. The major quinonic phenol compound of TQ in *Nigella sativa* L. seeds was quantified in $SCCO_2$ extracts and conventional solvent extracts. Subsequently, the $SCCO_2$ extracts and the essential oil recovered by hydrodistillation of $SCCO_2$ extract were subjected to further chemical characterization studies using GC, GC-MS and NMR spectroscopy.

$SCCO_2$ extraction was carried out at a pressure of 280 bar and a temperature of 50°C resulted in a total extract yield of 26.02% containing both volatiles and non volatile fractions. Whereas the $SCCO_2$ extract obtained at lower pressure and at suitable temperature extraction conditions was found to contain only a major fraction of volatile compounds (Udaya Sankar 1989).

Bioactive compounds

"Bioactive compounds" are extra nutritional constituents that typically occur in small quantities in natural sources like plants. They are being intensively studied to

evaluate their effects on health. The impetus sparking this scientific inquiry was the result of many epidemiological studies that have shown protective effects of plant based diets on many diseases and ailments like cancer. Bioactive compounds which have been discovered vary widely in their chemical structure and function and hence are grouped accordingly. Phenolic compounds, including their subcategory, flavonoids, are present in all plants and have been studied extensively in cereals, legumes, nuts, oils, vegetables, fruits, tea, and red wine.

Many phenolic compounds owing to their biological properties have demonstrated favorable influences on food and pharmaceutical industries. Resveratrol, which inhibits carcinogenesis by its antioxidant, antithrombotic, and anti-inflammatory properties, found rich in nuts and red wine. Lycopene, a potent antioxidant carotenoid present in tomatoes and other fruits provides protection against prostate and other cancers, inhibits tumor cell growth in animals. Organosulfur compounds in garlic and onions, isothiocyanates in cruciferous vegetables, and monoterpenes in citrus fruits, cherries, and herbs have anticarcinogenic actions in experimental models, as well as cardio protective effects (Kris-Etherton *et al.* 2002).

Based on these reports, it is clear that numerous bioactive compounds present in plant materials appear to have beneficial health effects. Hence, extensive scientific research work needs to be carried out prior to dietary recommendations for consuming foods rich in bioactive compounds.

Quinonic phenol compounds

Currently, plant seeds have been extensively studied and particularly *Nigella sativa* seed is being explored for its broad spectrum of traditional therapeutic and nutritional values. Additionally with influences in folk medicine, *Nigella sativa* seed appears to be a promising source for bioactive ingredients that has potential

therapeutic modalities for different clinical practices. Major quinonic phenol compounds like thymoquinone (TQ), dithymoquinone (DTQ), thymohydroquinone (THQ) and thymol (THY) (Figs. 3.1-3.4 and Tables 3.1-3.3) have been identified in the seed (Morikawa *et al.* 2004). Though, *Nigella sativa* seed is a complex substance of more than one hundred constituents, some of which have not yet been identified or studied (Salem 2005).

Thymoquinone (TQ)

IUPAC Name **:** p-Mentha-3, 6-diene-2, 5-dione**;** Thymolquinone

CAS Registry Number **:** 490-91-5

Molecular Formula **:** $C_{10}H_{12}O_2$

Molecular Weight **:** 164.2011

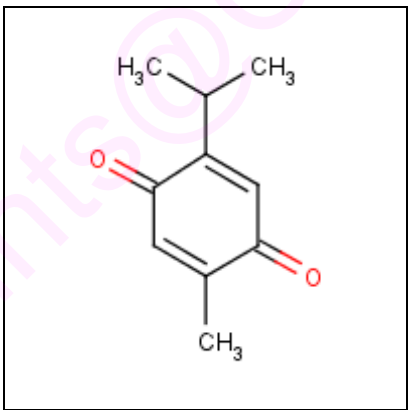


Fig. 3.1. Structure of thymoquinone.

Table 3.1. Physical properties of Thymoquinone.

S. No.	Physical Property	
1.	***Melting Point:***	45.5°C
2.	***Boiling Point:***	232°C
3.	***Solubility***	**a) Very sol:** EtOH, Et_2O **b) Sol:** $CHCl_3$, $C6H_6$ **c) Sparingly sol:** H_2O
4.	***Volatility:***	volatile in steam
5.	***Photodimerization***	**In light** Thymoquinone → Dithymoquinone **EtOH + light** Thymoquinone → Thymohydroquinone **Reduction** Thymoquinone → Thymohydroquinone

Dithymoquinone (DTQ)

Molecular Formula : $C_{20}H_{24}O_4$

Molecular Weight : 328

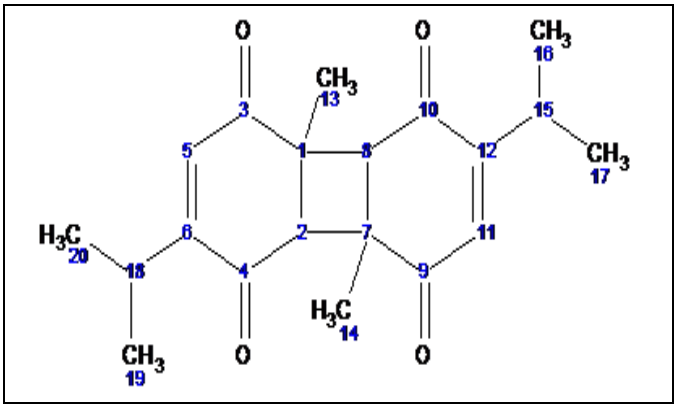


Fig. 3.2. Structure of dithymoquinone.

Thymohydroquinone (THQ)

IUPAC Name : 2-methyl-5-(1-methylethyl)-2-Methyl-5-isopropylhydroquinone; Thymoquinol

CAS Registry Number : 2217-60-9

Molecular Formula : $C_{10}H_{14}O_2$

Molecular Weight : 166.2170

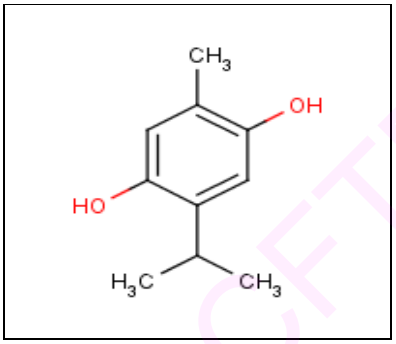


Fig. 3.3. Structure of Thymohydroquinone.

Table 3.2. Physical properties of Thymohydroquinone.

S. No.	Physical Property	
1.	***Melting Point:***	143°C (139.5°C)
2.	***Boiling Point:***	290°C
3.	***Solubility***	**a) Very sol:** EtOH, Et_2O **b) Sol:** Hot water **c) Insol:** $C6H_6$
4.	***Oxidation***	Thymohydroquinone → Thymoquinone

Thymol (THY)

IUPAC Name : Thyme camphor; 1-Methyl-3-hydroxy-4-isopropylbenzene

CAS Registry Number : 89-83-8

Molecular Formula : $C_{10}H_{14}O$

Molecular Weight : 150.2176

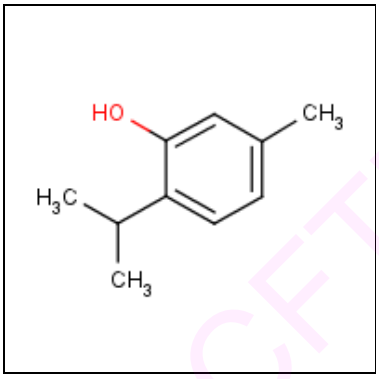


Fig. 3.4. Structure of Thymol.

Table 3.3. Physical properties of Thymol.

S. No.	Physical Property	
1.	***Melting Point:***	51.5°C
2.	***Boiling Point:***	233.5°C
3.	***Solubility***	**a) Very sol:** C_6H_6 **b) Sol:** Glacial acetic acid, oils and fixed alkali hydroxides. **c) Sparingly sol:** 1 gram dissolves in approx. 1000 ml H_2O, 1ml alcohol, 0.7 ml $CHCl_3$, 1.5 ml Et_2O and 1.7 ml olive oil at 25°C.
4.	***Oxidation***	Thymol → Thymohydroquinone or Dithymol

(Dictionary of organic compounds 1953; The Merck index 2006).

The aim of the present study was to explore the chemical composition of extracts isolated from *Nigella sativa* seeds by Supercritical CO_2 besides identifying all the compounds present in the volatile oils by GC and GC-MS and NMR spectroscopy. The purified fractions from the $SCCO_2$ extract were subjected to characterization studies to deduce and identify the presence of four major quinonic phenol compounds. Further, the $SCCO_2$-2 extract (obtained at conditions of 280 bar pressure and 50°C of temperature) was purified by column chromatography and the fractions obtained were subjected to spectral characterization studies along with 2D HSQCT NMR spectroscopy for isolation and characterization of major bioactive compound.

3.2. Materials and methods

Plant Material

Seeds of *Nigella sativa* were obtained from Supreem Pharmaceuticals Mysore Pvt. Ltd, Mysore, India. A voucher specimen authenticated has also been deposited at the Central Food Technological Research Institute, Mysore. The seeds were stored in polythene bags and maintained at 4°C until extraction. The seed material was dried and ground into a fine powder using an IKA-Analytical Mill A10 (Janke & Kunkel GmbH & Co. KG, Germany).

Chemicals

Sodium sulphate (anhydrous) and silica gel (60-120 mesh) for column chromatography and silica gel G for thin layer chromatography were purchased from SD Fine Chemicals, India. Standard Thymoquinone was obtained from Sigma-Aldrich, USA. All the solvents used were of analytical grade and were obtained along with dimethyl sulfoxide (*DMSO-d_6*) and chloroform (*$CDCl_3$*) from Merck Co, Mumbai, India. The solvents were distilled once before use. For the determination of retention indices, a hydrocarbon mixture (Sigma, India) ranging from *n*-octane to *n*-docosane was used. Food grade CO_2 cylinder (of 99.9% purity) was obtained from Kiran Corporation, Mysore, India.

Supercritical fluid extraction

High pressure extractor (Nova Werke, AG, model Ex 1000-1.4-1.2) was employed for the extraction of *Nigella sativa* seeds as already described in Chapter 2 under section 2.2. The extractions were carried out at two different conditions of pressures and temperatures, namely optimized condition at 120 bar at 40°C as major volatile part ($SCCO_2$-1) and 280 bar at 50°C as total extract ($SCCO_2$-2) at a CO_2 mass flow rate of 3.30-8.05 $\times 10^{-4}$ kg/s.

Hydrodistillation

The supercritical CO_2 extract ($SCCO_2$-2) was subjected to hydrodistillation (HD) for 6 h using a '*Clevenger-type*' apparatus. The essential oil (HD $SCCO_2$) obtained was yellow in color with an aromatic odor and an yield of 1.5% (v/v), which was dried using anhydrous sodium sulphate and then stored at 4°C in dark until further analysis.

Chemical composition of black cumin seed extracts

The chemical composition of *Nigella sativa* seed extracts were analysed by GC, GC-MS and NMR spectroscopy.

GC and GC-MS analysis

GC analyses were performed using a gas chromatograph (Fisons GC 8000 series, USA) equipped with Flame ionization detector (FID). All the analyses were carried out by a fused silica DB-5MS column (30 m × 0.32 mm i.d., film thickness 0.25µm). The oven temperature was increased from 70°C to 220°C at an increment of 4°C per min and held isothermal for 15 min. The injector and detector temperatures were maintained at 220°C and 240°C, respectively. Samples were prepared at 10% concentration with chloroform. The injection volume was 0.5µL with a split ratio of 1:30 and nitrogen was used as carrier gas at a flow rate of 1 ml min^{-1}. GC-MS analyses were made on a gas chromatograph coupled with QP-5000 mass spectrometer (Shimadzu, Japan). A 0.5µL sample was injected in the split mode ratio of 1:15. Helium was used as carrier gas at a flow rate of 1 ml min^{-1}. All the other parameters remained unchanged relative to GC analyses. Mass spectra were obtained by EI at 70 eV. Mass scanning was performed from 40 to 400 amu.

Compounds identification

The compounds were identified based on comparison of their Kovats retention indices (RI) relative to C_8-C_{22} *n*-alkanes arrived at using GC-MS studies and matching of the mass spectra with those detailed in the NIST, Wiley commercial libraries, data from Chemistry web book and literature data (Joulain and Konig 1998; Adams 2007).

Major quinonic phenol compounds

The occurrence of major quinonic phenol compounds in the column chromatographed fractions of total extract ($SCCO_2$-2) was confirmed by 2D HSQCT ^1H and ^{13}C NMR spectroscopy.

Column chromatography

5 ml of $SCCO_2$-2 extract was subjected to purification, using a glass column (40 mm i.d × 450 mm length) packed with silica gel (60-120 mesh) and eluted with hexane and ethyl acetate at 99:1 ratio at a flow rate of 1 ml/min. Fractions of volume 250 ml were collected and concentrated. They were monitored by TLC in 9:1 ratio of hexane and ethyl acetate. The spots were located by exposing the plates to iodine vapours. Fractions having similar pattern of spots and Rf values on the TLC plates were pooled and concentrated by using rotary vacuum evaporator (40°C). From chromatographic separation, four fractions were detected. The fraction volumes/weights were: fraction 1; 1.3 litre, fraction 2; 0.8 L, fraction 3; 0.4 L and fraction 4; 0.7 L.

2D HSQCT ^1H and ^{13}C NMR analysis

Two-dimensional Heteronuclear Single Quantum Coherence Transfer Spectra (2D HSQCT) were recorded using a Bruker Avance AQS 500 MHz (Bruker Biospin, Fallanden, Switzerland) NMR spectrometer operating at 500.18 MHz for ^1H and 125.78 MHz for ^{13}C at 20°C. Proton and carbon 90° pulse widths were 12.25 and 10.5

μs, respectively. Chemical shifts were expressed in ppm relative to tetramethylsilane (TMS) as an internal standard. Samples of 5mg dissolved in DMSO-d_6 were used for recording the spectra in magnitude mode with sinusoidal-shaped z-gradients of strength 25.7, 15.42 and 20.56 G/cm with a gradient recovery delay of 100μs to defocus unwanted coherences. The increment of *t*1 was in 256 steps. About 50-200 scans and 500-6000 scans were accumulated with a recycle period of 2-3 seconds to obtain good spectra for ^{1}H and ^{13}C NMR, respectively. A region from 0-10 ppm and 0-200 ppm were scanned for all the samples for ^{1}H and ^{13}C NMR, respectively. The size of the computer memory used to accumulate the data was 4 kB. The spectra were processed using unshifted and π/4 shifted sine bell window function in F1 and F2 dimensions, respectively.

Isolation and characterization of major bioactive compound

In the present study, $SCCO_2$-2 extract (as total extract obtained at conditions of 280 bar pressure and 50°C of temperature) was further purified through column chromatography as already described in this Chapter and the obtained fractions were subjected to spectral characterization studies along with 2D HSQCT ^{1}H and ^{13}C NMR analysis using *$CDCl_3$* as solvent for the isolation and characterization of major bioactive compound.

Isolation of major bioactive compound

Silica gel-column chromatography

Activated silica gel (60-120 mesh) was packed onto a glass column (40 × 450 mm) using *n*-hexane solvent. 10 ml of $SCCO_2$-2 extract was subjected to purification. The column was eluted at a flow rate of 0.5 ml/min with hexane and ethyl acetate at 99:1 ratio. The fractions were collected.

Thin-layer chromatography

Collected fractions were monitored by silica gel-G coated TLC plates as per the method explained under column chromatography.

High-performance liquid chromatography

The purity of isolated compound was checked by RP-HPLC DAD analysis and the elution conditions employed were similar as mentioned in Chapter 2.

Spectral characterization of isolated compound

The isolated compound obtained was subject to various spectral characterization studies. The isolated compound besides being measured for melting point was characterized spectroscopically by UV, IR, Mass and Two-Dimensional Heteronuclear Single Quantum Coherence Transfer (2D-HSQCT) NMR spectra.

UV-visible spectroscopy

Ultraviolet (UV)-visible spectrum of the isolated compound was recorded on a UV-160A spectrophotometer (Shimadzu, Japan). About 1mg of the isolated compound dissolved in 20ml of methanol was used to obtain the spectrum (200-800nm).

IR spectroscopy

Infra-red (IR) spectrum was recorded on a Perkin-Elmer FT-IR spectrometer. About 1 mg of the isolated compound was prepared as KBr pellets and employed for recording the IR spectrum (frequencies between 4000 and 400 cm^{-1}).

Mass spectroscopy

Mass spectrum of the isolated compound was recorded using a Q-TOF Waters Ultima mass spectrometer (Waters Corporation, Manchester, UK) fitted with an electro spray ionization (ESI) source with negative ionization mode. The mass spectrometry conditions were as follows: capillary voltage of 3.5 kV, source

temperature of 120°C, cone voltage of 100 V, dissolvation temperature of 300°C and the cone and dissolvation gas flow were at 50 L/hr and 500 L/hr, respectively. The scanning range of 100-400 m/z with a scan speed of 1000 amu/sec was employed. About 1 mg of the isolated compound was dissolved in 5 ml of methanol and was injected for recording the mass. Liquid chromatography conditions were similar to reverse-phase HPLC analysis, as described in Chapter 2.

2D-HSQCT NMR spectroscopy

2D HSQCT was recorded along with ^{1}H and ^{13}C NMR spectrum on a Bruker Avance AQS-500 MHz (Bruker Biospin, Fallanden, Switzerland) NMR spectrometer (500.13 MHz for ^{1}H and 125 MHz for ^{13}C) at 20°C using 5 mg of the isolated compound in $CDCl_3$ solvent. A region from 0-10 ppm for ^{1}H and 0-200 ppm for ^{13}C were scanned. Chemical shift values were expressed in ppm relative to internal tetramethyl silane (TMS) as the standard. In the NMR data, only resolvable signals are shown. Some of the assignments are interchangeable. Certain quaternary carbons and proton signals could not be detected.

Statistical analysis

The experiments were performed in triplicate and the data were calculated as means ± SD. A probability value of $P < 0.05$ was considered as significant.

3.3. Results

The $SCCO_2$-1 extract was found to contain a major fraction of volatile components only at lower pressure and temperature of extraction conditions. Whereas the $SCCO_2$-2 extraction carried out at a pressure of 280 bar and a temperature of 50°C resulted in a total extract yield of 33.0%, containing both volatile and nonvolatile fractions. In the present study, the extract yield is expressed in % and defined as weight of the extract divided by weight of the sample. $SCCO_2$-2 obtained higher extract yield than the $SCCO_2$-1. The extraction yield was increased with increasing pressure and temperature to a particular level, but yield of quinones and phenolics were less. The hydrodistillation of *Nigella sativa* seed powder with Clevenger distillation yielded 1.0-1.2% of essential oil with 30-32% of thymoquinone (TQ). In $SCCO_2$-1, the extract yield was 16.2% with 12.3% of TQ whereas in $SCCO_2$-2, the extract yield was 33.0% with TQ content of 7.1%. TQ extracted on the seed basis was 0.4-0.5% in hydrodistillation, 1.99% in $SCCO_2$-1 and 2.34% in $SCCO_2$-2 extracts. Hence, by $SCCO_2$ extraction the recovery of TQ was much better.

Hydrodistillation of $SCCO_2$-2 extract yielded 1.5% of essential oil (HD $SCCO_2$) with a characteristic transparent yellow color and a typical aromatic odour. A total of 47 different compounds were identified in *Nigella sativa* seed oils extracted by supercritical CO_2 ($SCCO_2$-1 and 2) and hydrodistillation of $SCCO_2$-2 (HD $SCCO_2$). About 22 compounds in $SCCO_2$-1, 31 compounds in $SCCO_2$-2 and 23 compounds in HD $SCCO_2$ extracts were identified. Hence, this might be the first report on the chemical composition of essential oil obtained by hydrodistillation of the $SCCO_2$ extract of *Nigella sativa* seeds. Among the 47 compounds, the occurrences of 16 volatile compounds were reported for the first time in *Nigella sativa* seeds (Table 3.4). They are *n*-nonane, *allo*-ocimenol, terpinen-1-ol, 1,5,8-ρ-menthatriene,

dihydrocarvone, ocimenone (E), n-octyl isobutyrate, citronellyl acetate, thymohydroquinone methyl ether, (Z)-caryophyllene, thymohydroquinone dimethyl ether, aromadendrene, davanone, 8-heptadecene, dihydro farnesyl acetate and pimaradiene. The lower number of compounds occurred in HD $SCCO_2$ compared to $SCCO_2$ is mainly related to the possible degradation of volatile compounds by higher temperature and longer distillation time.

Table 3.4. Chemical composition (%) of black cumin seed extracts isolated by supercritical CO_2 extraction.

Compound	RI_{exp}	RI_{lit}	$SCCO_2$-1	$SCCO_2$-2	HD $SCCO_2$	Identification
n-Nonane*	905	900	—	0.12	—	RI, MS
Tricyclene	926	926	—	tr	—	RI, MS
Camphene	953	953	—	—	1.64	RI, MS
β-Pinene	958	959	—	—	0.40	RI, MS
2,4,(10)-Thujadiene	967	960	0.19	4.74	—	RI, MS
Sabinene	978	977	—	1.05	—	RI, MS
β-Myrcene	990	991	—	0.31	—	RI, MS
1,8-Cineole	1013	1010	—	—	0.98	RI, MS
α-Terpinene	1025	1026	—	2.34	—	RI, MS
Limonene	1034	1034	0.38	0.18	1.03	RI, MS
γ-Terpinene	1054	1056	13.20	27.46	12.87	RI, MS
cis-Sabinene hydrate	1063	1068	0.38	—	tr	RI, MS
allo-Ocimenol*	1079	1071	0.11	—	—	RI, MS
Linalool	1087	1080	0.19	0.25	—	RI, MS
Terpinolene	1091	1088	—	—	tr	RI, MS
trans-Sabinene hydrate	1099	1097	—	0.37	—	RI, MS
Terpinen-1-ol*	1124	1120	—	—	0.11	RI, MS
1,5,8-p-Menthatriene*	1130	1135	0.38	0.43	—	RI, MS
Borneol	1152	1152	—	—	1.02	RI, MS
Pinocarvone	1167	1165	3.00	2.96	—	RI, MS
trans-Dihydrocarvone	1208	1202	0.19	—	—	RI, MS
Dihydrocarvone*	1215	1214	2.06	0.37	—	RI, MS
Ocimenone (E)*	1249	1239	1.50	1.54	—	RI, MS
Thymoquinone	1250	1250	33.12	35.05	38.41	RI, MS, NMR
Thymol	1283	1288	5.30	7.43	16.95	RI, MS, NMR
Carvacrol	1299	1299	1.73	1.98	0.81	RI, MS
2-Undecanone	1312	1315	—	—	13.72	RI, MS
n-Octyl isobutyrate*	1323	1326	—	—	0.12	RI, MS
α-Longipinene	1330	1334	—	0.26	—	RI, MS
Citronellyl acetate*	1339	1336	—	—	0.50	RI, MS
Thymohydroquinone methyl ether*	1353	1351	—	—	tr	RI, MS
Cyclosativene	1367	1366	—	—	1.43	RI, MS
α-Longicyclene	1381	1380	5.25	0.43	—	RI, MS

α-Copaene	1385	1383	2.00	1.54	0.41	RI, MS
α-Longifolene	1391	1387	—	—	0.51	RI, MS
(Z)-Caryophyllene*	1395	1395	—	0.23	—	RI, MS
β-Caryophyllene	1420	1417	5.07	2.89	4.80	RI, MS
Thymohydroquinone dimethylether*	1429	1425	—	0.43	—	RI, MS
Aromadendrene*	1437	1438	—	—	1.04	RI, MS
Thymohydroquinone	1515	1509	1.12	1.17	2.31	RI, MS, NMR
Davanone*	1587	1586	—	0.31	—	RI, MS
8-Heptadecene*	1683	1680	1.13	1.23	0.86	RI, MS
Dihydrofarnesyl acetate*	1841	1840	4.69	2.28	—	RI, MS
Pimaradiene*	1934	1935	2.25	1.23	—	RI, MS
Palmitic acid	1947	1946	—	0.18	—	RI, MS
Pimara-8(14),15-diene	1968	1966	—	0.92	—	RI, MS
Octadecanoic acid	2145	2157	12.31	0.26	—	RI, MS
Total identified						
Grouped compounds:			95.55	99.94	99.92	
Quinones			39.54	44.08	57.67	
Monoterpene hydrocarbons			14.15	36.51	15.94	
Oxygenated monoterpenes			9.16	7.47	17.14	
Sesquiterpene hydrocarbons			12.32	5.35	8.19	
Oxygenated sesquiterpenes			4.69	2.59	—	
Diterpenes			2.25	2.15	—	
Alkane			—	0.12	—	
Alkenes			1.13	1.23	0.86	
Fatty acids			12.31	0.44	—	
Fatty acid esters			—	—	0.12	

Identification has been through by comparing mass spectra (MS), retention indices (RI), NMR spectra, data from NIST (National Institute of Standards and Technology), Wiley commercial libraries, Chemistry Web Book (http://www.nist.org/chemistrywebbook) and other reports (Joulain and Konig 1998; Adams 2007). $SCCO_2$-1 (120 bar, 40°C), $SCCO_2$-2 (280 bar, 50°C) and HD $SCCO_2$ (hydrodistillation of $SCCO_2$-2). The retention indices were calculated for all compounds using a homologous series of C_8-C_{22} *n*- alkanes. RI_{exp}, experimental retention indices given for DB-5MS column; RI_{lit}, literature retention indices given for DB-5MS column. tr, trace (< 0.1%).
* Compounds identified for the first time in the extracts of *Nigella sativa*.

The $SCCO_2$ oils could be distinguished from the HD $SCCO_2$ oil by their greater richness in monoterpene hydrocarbons (14.15% in $SCCO_2$-1, 36.51% in $SCCO_2$-2 and 15.94% in HD $SCCO_2$), sesquiterpene hydrocarbons (12.32% in $SCCO_2$-1, 5.35% in $SCCO_2$-2 and 8.19% in HD $SCCO_2$), oxygenated sesquiterpenes (4.69% in $SCCO_2$-1 and 2.59% in $SCCO_2$-2), diterpenes (2.25% in $SCCO_2$-1 and 2.15% in $SCCO_2$-2), fatty acids (12.31% in $SCCO_2$-1 and 0.44% in $SCCO_2$-2) and fatty acid esters (0.12% in HD $SCCO_2$). The oxygenated monoterpenes were more represented in HD $SCCO_2$ (17.14%) than in $SCCO_2$-1 (9.16%) and $SCCO_2$-2 (7.47%). Quinones were present to a greater extent in HD $SCCO_2$ (57.67%) than in $SCCO_2$-1 (39.54%) and $SCCO_2$-2 (44.08%) oils. The main compounds in $SCCO_2$-1, $SCCO_2$-2 and HD $SCCO_2$ oils were thymoquinone (33.12% in $SCCO_2$-1, 35.05% in $SCCO_2$-2 and 38.41% in HD $SCCO_2$), γ-terpinene (13.20% in $SCCO_2$-1, 27.46% in $SCCO_2$-2 and 12.87% in HD $SCCO_2$), thymol (5.30% in $SCCO_2$-1, 7.43% in $SCCO_2$-2 and 16.95% in HD $SCCO_2$), β-caryophyllene (5.07% in $SCCO_2$-1, 2.89% in $SCCO_2$-2 and 4.80% in HD $SCCO_2$) and thymohydroquinone (1.12% in $SCCO_2$-1, 1.17% in $SCCO_2$-2 and 2.31% in HD $SCCO_2$). In this present study, dithymoquinone (DTQ) could not be traced by GC and GC-MS analysis of these oils.

The fractions obtained from column chromatography separation of $SCCO_2$-2, showed a mixture of four quinonic phenol compounds, all possessing the thymol skeleton through 2D HSQCT NMR spectra in different proportions as follows:

Compound 1 showed the presence of two carbonyl groups at 187.3 ppm and 188.3 ppm. Further, two aromatic protons were detected at 6.59 ppm and 6.72 ppm. A CH_3 group attached to an aromatic ring was detected at 14.9 ppm in the carbon spectrum. The presence of isopropyl group was detected by observing signals at 1.93

ppm and 1.94 ppm (doublet) and 2.12 ppm, all with the coupling constant of 5.3 Hz (Table 3.5). Thus, compound 1 was identified to be thymoquinone (Fig. 3.5).

Compound 2 showed methyl groups attached to an aromatic ring and an isopropyl group attached to an aromatic ring at 14.3 ppm and 31.1 ppm (^{1}H 1.92 ppm), 24.5 ppm (^{1}H 1.41 ppm), 25.3 ppm (^{1}H 1.47 ppm) respectively with a characteristic coupling constant value of 5.1 Hz. Two aromatic protons were detected at 5.30 ppm and 7.03 ppm (Table 3.5). All these showed that compound 2 could be thymohydroquinone (Fig. 3.5).

Compound 3 showed the characteristic thymol ^{1}H and ^{13}C NMR characteristics. Two ortho coupled signals were detected at 7.47 ppm and 7.85 ppm with a characteristic ortho coupling constant of 8.0 Hz. A single aromatic peak was also detected at 6.72 ppm. Phenolic carbon was observed at 154.1 ppm. An isopropyl group attached to an aromatic ring (30.8 ppm, 23.5 ppm and 26.2 ppm) was also observed (Table 3.5). Thus, compound 3 clearly showed the characteristics of thymol (Fig. 3.5).

Two dimentional NMR characteristics of compound 4 showed clearly fusion of two thymoquinone rings with two alicyclic methyl groups (at 1.07 ppm and 1.06 ppm) along with two isopropyl groups attached to the quinone ring. Only two aromatic protons were detected at 5.4 ppm. Four keto groups were detected at 172.9 ppm and 192.7 ppm clearly indicating the fusion of two thymoquinone moieties (Table 3.6). Thus, compound 4 was identified to be dithymoquinone (Fig. 3.5).

Table 3.5. ^{1}H and ^{13}C 2D-HSQCT NMR data of compound 1, 2 and 3[a] in DMSO-d_6

Carbon number	Chemical shifts (ppm) Compound 1		Chemical shifts (ppm) Compound 2		Chemical shifts (ppm) Compound 3	
	^{1}H NMR (J in Hz)	^{13}C NMR	^{1}H NMR (J in Hz)	^{13}C NMR	^{1}H NMR (J in Hz)	^{13}C NMR
1	—	145.1	—	127.2	—	—
2	6.59 (s)	133.5 (i1)	7.03 (s)	120.4	6.72 (s)	133.5 (i1)
3	—	188.3 (i2)	—	—	7.47 (d, 8 Hz)	127.9 (i2)
4	—	187.3 (i2)	—	—	—	154.1
5	6.72 (s)	133.3 (i1)	5.30 (s)	129.3	7.85 (d, 8 Hz)	125.8 (i1)
6	—	156.4	—	133.5	—	127.7 (i2)
7	—	14.9	—	14.3	—	14.2
8	2.12 (m, 5.3 Hz)	31.0	1.92 (m, 5.1 Hz)	31.1	2.01 (m, 5.4 Hz)	30.8
9	1.93 (d, 5.3 Hz)	26.7	1.41 (d, 5.1 Hz)	24.5	1.37 (d, 5.4 Hz)	23.5
10	1.94 (d, 5.3 Hz)	26.7	1.47 (d, 5.1 Hz)	25.3	1.41 (d, 5.4 Hz)	26.2

[a]5 mg of sample was dissolved in 0.5 ml of DMSO-d_6 for recording the spectra at 20°C on Bruker Avance AQS 500 MHz NMR spectrometer. All the details are mentioned in Materials and Methods. s-singlet, d-doublet, m-multiplet and i-interchangeable.

Table 3.6. ^{1}H and ^{13}C 2D-HSQCT NMR data of compound 4[a] in DMSO-d_6

Carbon number	Chemical shifts (ppm) Compound 4	
	^{1}H NMR (J in Hz)	^{13}C NMR
1	—	59.7 (i1)
2	4.59 (s)	59.4 (i1)
3	—	172.9 (i2)
4	—	192.7 (i2)
5	5.40 (s)	129.8 (i3)
6	—	154.1 (i4)
7	—	59.6 (i1)
8	4.02 (s)	59.4 (i1)
9	—	172.9 (i2)
10	—	192.7 (i2)
11	5.40 (s)	130.3 (i3)
12	—	153.0 (i4)
13	1.07 (s)	22.0 (i5)
14	1.06 (s)	20.9 (i5)
15	2.27 (m, 5.3 Hz)	33.6
16	1.18 (d, 5.3 Hz)	29.2
17	1.17 (d, 5.3 Hz)	29.1
18	2.26 (m, 5.5 Hz)	33.4
19	1.16 (d, 5.5 Hz)	28.6
20	1.15 (d, 5.5 Hz)	28.5

[a]5 mg of sample was dissolved in 0.5 ml of DMSO-d_6 for recording the spectra at 20°C on Bruker Avance AQS 500 MHz NMR spectrometer. All the details are mentioned in Materials and Methods. s-singlet, d-doublet, m-multiplet and i-interchangeable.

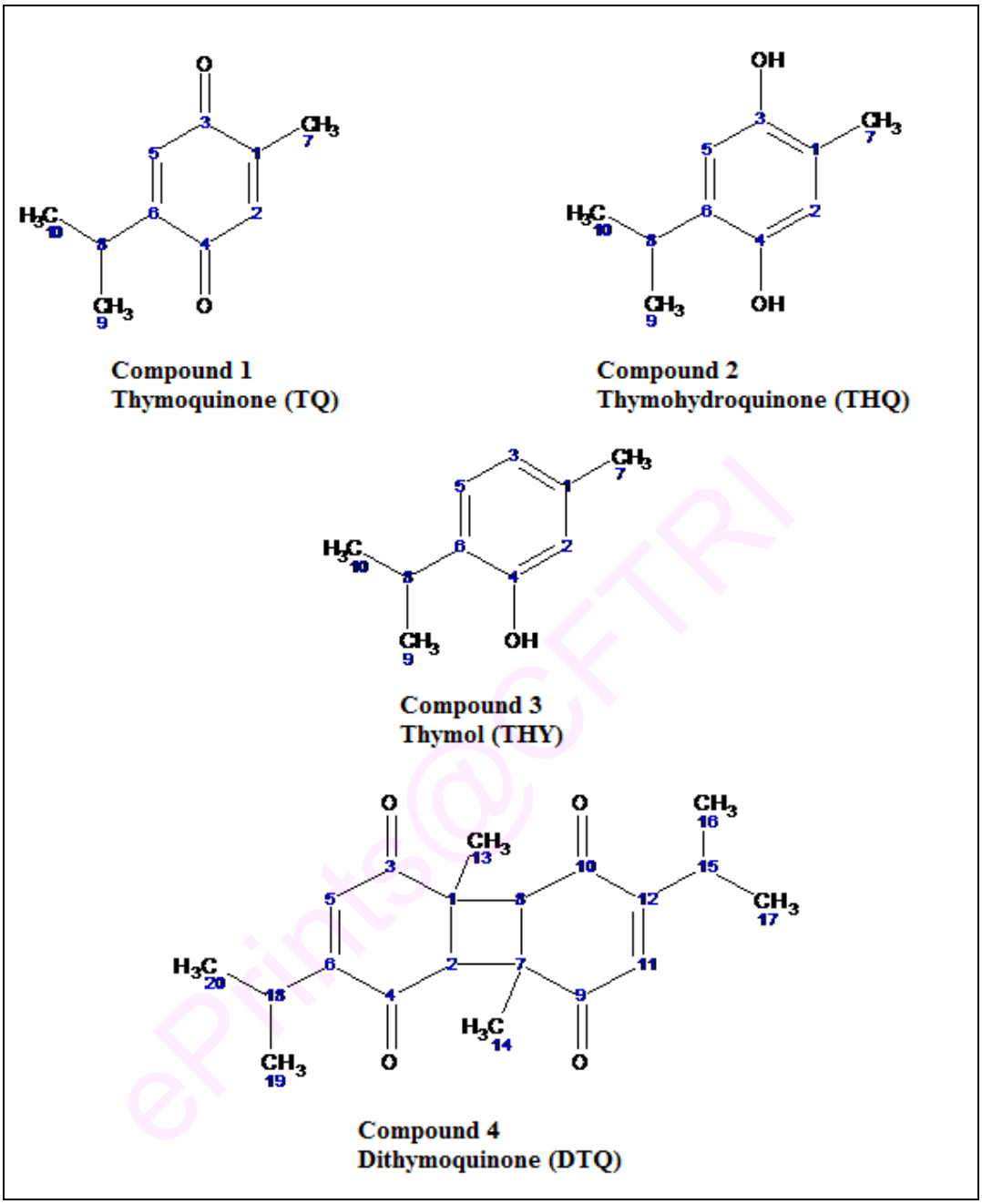


Fig. 3.5. Structures of thymoquinone, thymohydroquinone, thymol and dithymoquinone.

Typical ESI (¯ ve) MS finger prints of the fractions obtained from column chromatographic separation of $SCCO_2$-2 extract showed good correspondence for an *m/z* values of thymoquinone, thymohydroquinone, thymol and dithymoquinone (Figs. 3.6—3.9).

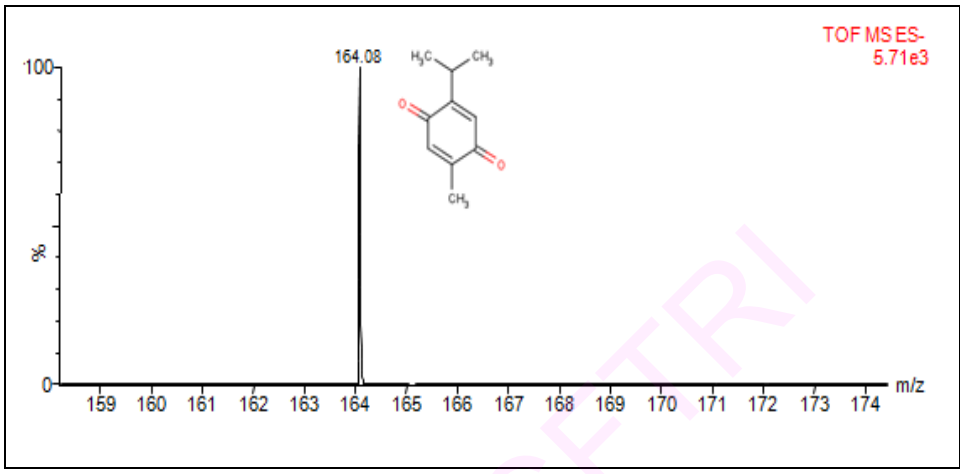


Fig. 3.6. ESI (— ve) MS finger print of thymoquinone (TQ).

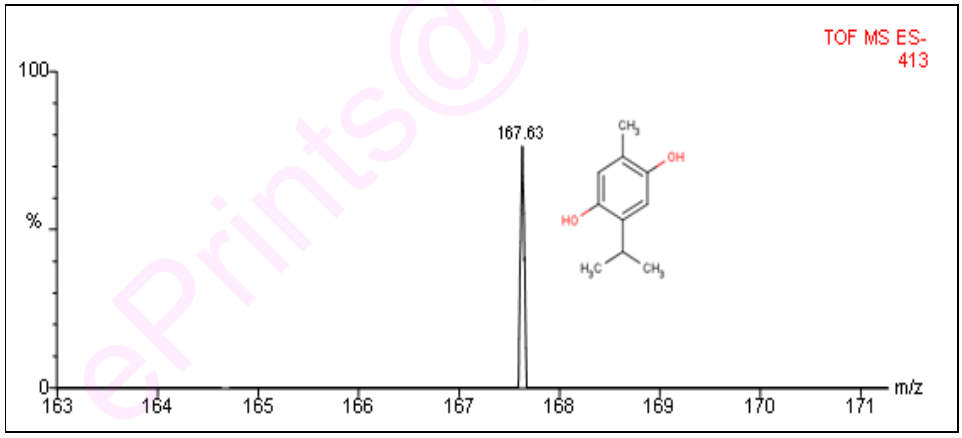


Fig. 3.7. ESI (— ve) MS finger print of thymohydroquinone (THQ).

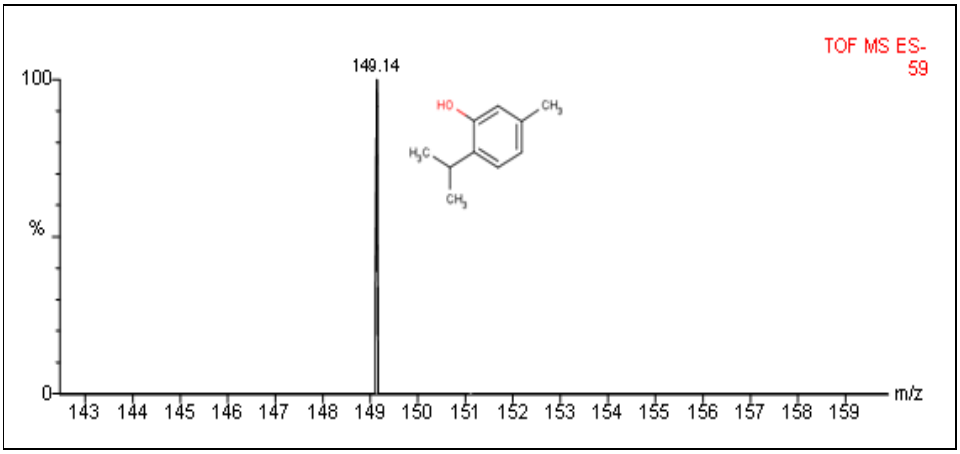


Fig.3.8. ESI (– ve) MS finger print of thymol (THY).

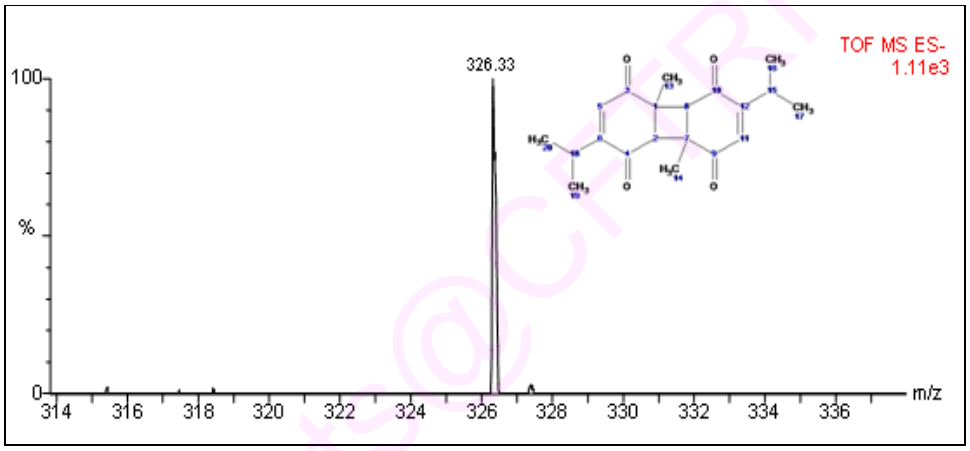


Fig. 3.9. ESI (– ve) MS finger print of dithymoquinone (DTQ).

Based on all these data which proved that the occurrence of thermally labile or photosensitive bioactive volatiles of four major quinonic phenol compounds of thymoquinone, thymohydroquinone, thymol and dithymoquinone in $SCCO_2$-2 extract. This study showed that HD $SCCO_2$ gives an essential oil of better quality especially in terms of richness in quinones including better recovery of main compounds of thymoquinone, γ-terpinene, thymol, β-caryophyllene and thymohydroquinone than the extracts obtained by $SCCO_2$ alone. It may be noted that ρ-cymene was not detected.

Isolation and characterization of major bioactive compound

The pure compound isolated from $SCCO_2$-2 (280 bar, 50°C) extract through column chromatography was characterized spectroscopically by UV, IR, Mass and Two-Dimensional Heteronuclear Single Quantum Coherence Transfer (2D HSQCT) NMR spectra to deduce the structure. Isolated compound and standard thymoquinone was detected in similar retention time at 254 nm in RP-HPLC-DAD analysis (Fig.3.10).

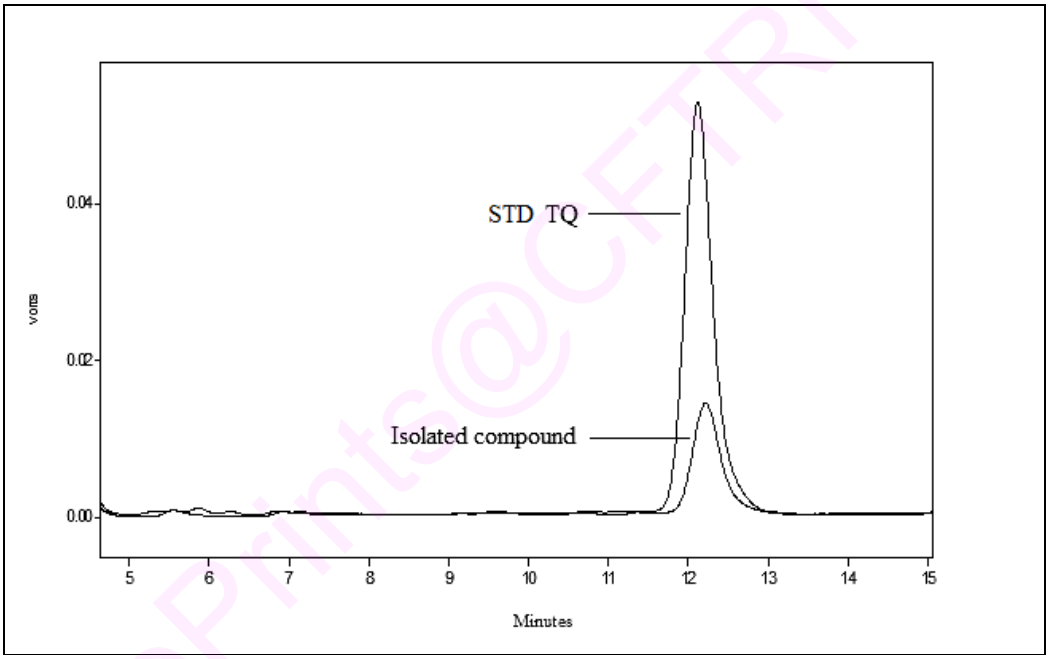


Fig. 3.10. RP-HPLC-DAD chromatogram of standard thymoquinone (STD TQ) and isolated compound from $SCCO_2$-2 extract of *Nigella sativa* L. seed detected at 254 nm.

The isolated compound was solid with melting point at 45.5°C and exhibited UV (λ_{max}): 278 nm ($\pi \rightarrow \pi^*$, ε_{278}–2420 M^{-1}). IR (KBr) spectral data showed C—H stretching at 2970 cm^{-1} and carbonyl stretching at 1690 cm^{-1} indicating the presence of alkyl CH and carbonyl groups respectively (Fig. 3.11). A typical ESI (— ve) MS

finger print of isolated compound showed molecular ion peak at 164.12 $[M]^+$ which resembles to the molecular weight of thymoquinone (Fig. 3.12).

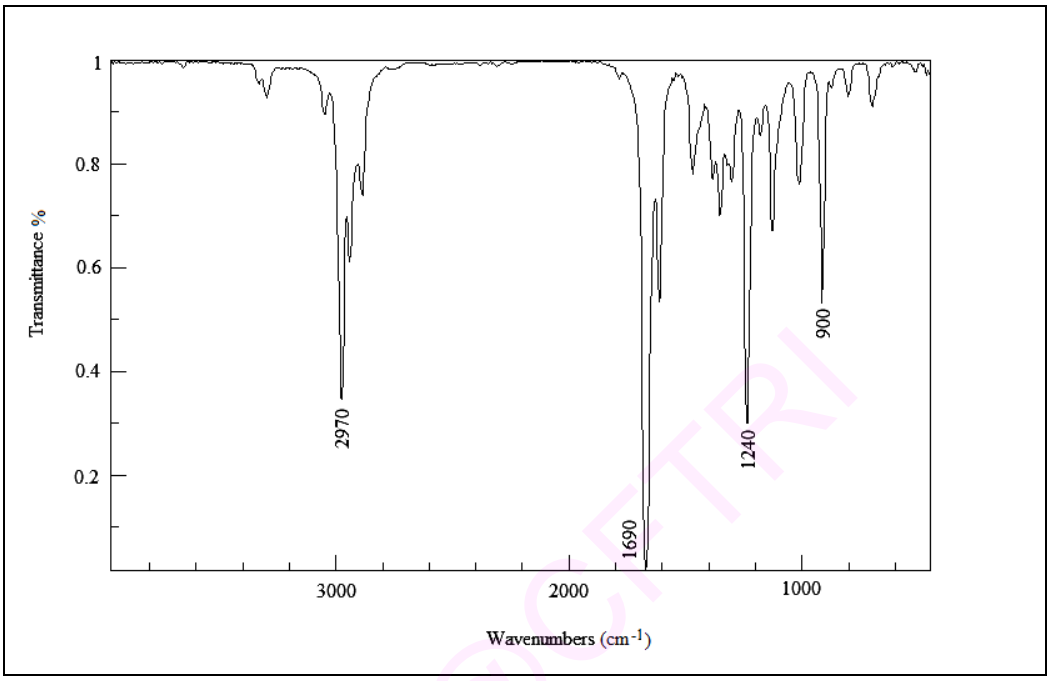


Fig. 3.11. IR (Infra-red) spectrum of isolated compound.

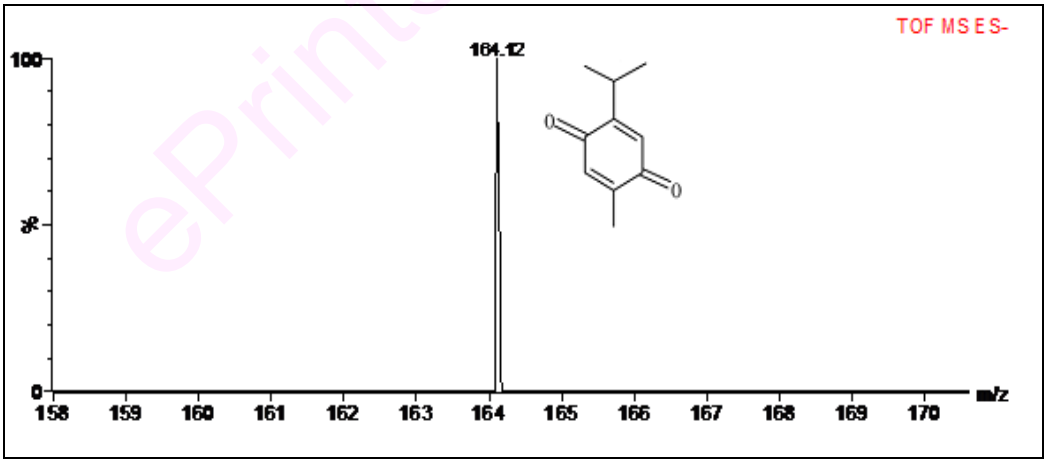


Fig. 3.12. ESI (— ve) MS finger print of isolated compound.

2-D HSQCT NMR spectra were recorded in $CDCl_3$ at 20°C on Bruker Avance AQS 500 MHz NMR spectrometer which showed that the isolated compound could be thymoquinone (Figs. 3.13, 3.14 and 3.15; Table 3.5).

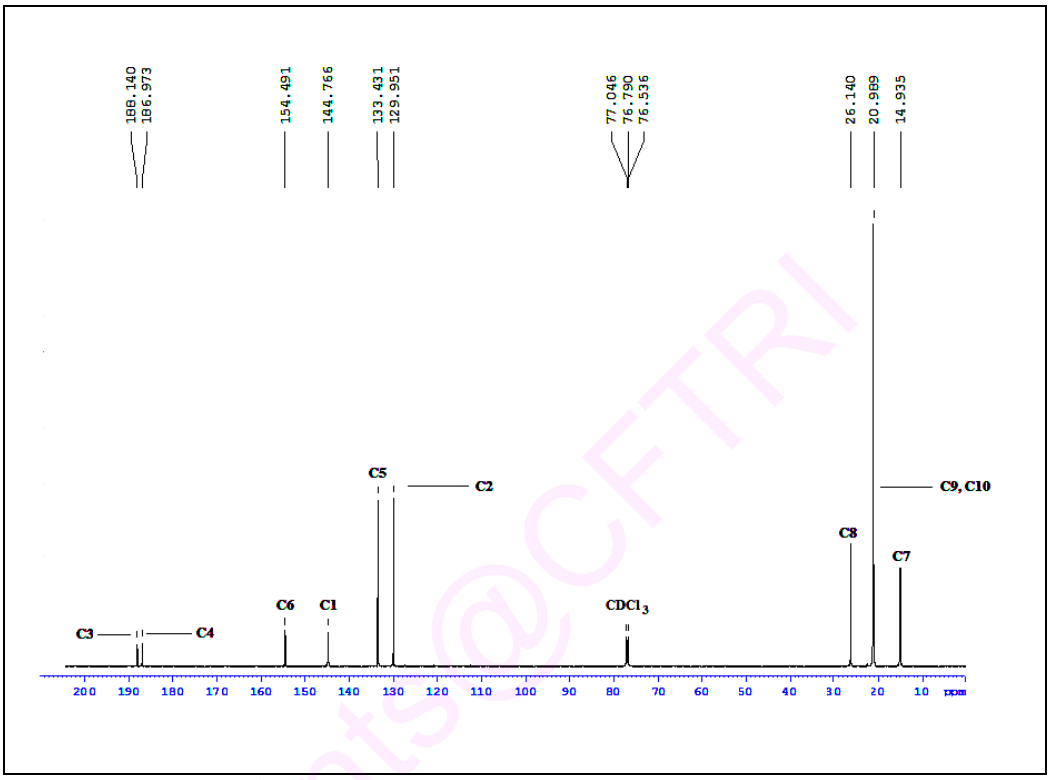


Fig. 3.13. ^{13}C NMR spectrum of isolated thymoquinone. Spectrum recorded in $CDCl_3$ at 20°C on Bruker Avance AQS 500 MHz NMR spectrometer.

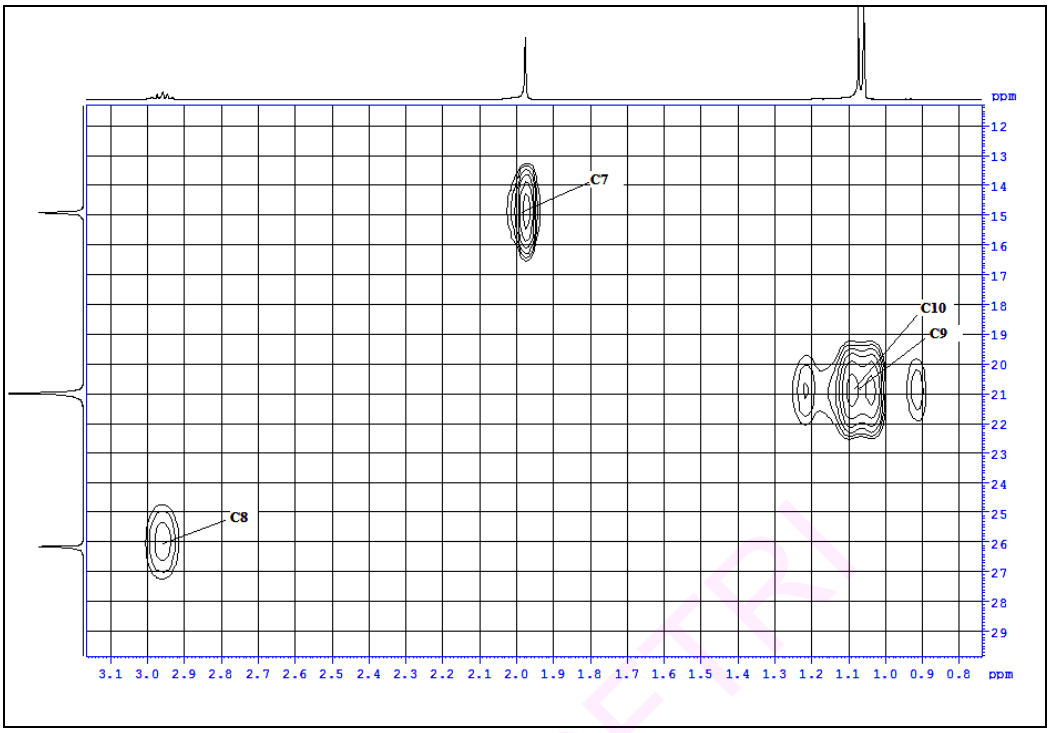


Fig. 3.14. 2D HSQCT spectrum of isolated thymoquinone showing the region between 0.1—3.1 ppm. Spectrum recorded in $CDCl_3$ at 20°C on Bruker Avance AQS 500 MHz NMR spectrometer.

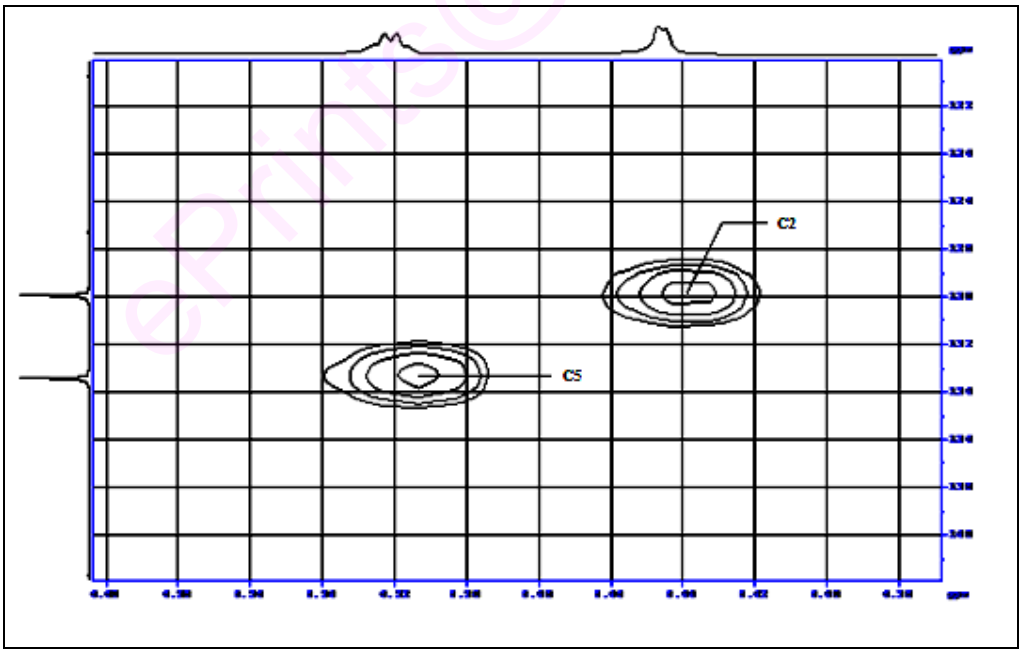
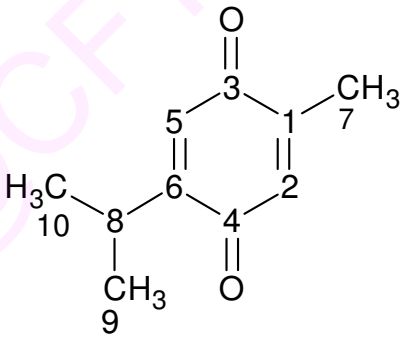


Fig. 3.15. 2D HSQCT spectrum of isolated thymoquinone showing the region between 6.37—6.60 ppm. Spectrum recorded in $CDCl_3$ at 20°C on Bruker Avance AQS 500 MHz NMR spectrometer.

Based on these above mentioned spectral characterization data, the isolated major bioactive compound structure was deduced and identified as thymoquinone (TQ) (Table 3.7).

Table 3.7. Spectral data for the isolated thymoquinone.

UV λ_{max} (Alcohol) 278 nm	
IR (KBr) data	2970 cm^{-1} (Alkyl—CH stretching), 1690 cm^{-1}(C=O stretching)
MS (*m/z*)	164.12 $[M]^+$
Structure	 Thymoquinone
Empirical formula	$C_{10}H_{12}O_2$

UV-Ultraviolet, IR- Infrared, MS- Mass spectroscopy

3.4. Discussion

The $SCCO_2$-1 extract found to contain a major fraction of volatile components only at lower pressure and temperature of extraction as reported earlier (Udaya Sankar 1989). In this study, dithymoquinone (DTQ) could not be traced by GC and GC-MS analysis of the extracted oils, as confirmed previously (Burits and Bucar 2000; Benkaci Ali *et al*. 2007). The present analysis showed higher thymoquinone content than it has been reported in the literatures so far (Burits and Bucar 2000; El Ghorab 2003). HD $SCCO_2$ (essential oil) yields better recovery of thymoquinone, γ-terpinene, thymol, β-caryophyllene and thymohydroquinone. More than 50% of the essential oil compounds of *Nigella sativa* are comprised of thymoquinone and ρ-cymene only (Machmudah *et al*. 2005). It is interesting to observe that ρ-cymene was not detected here because the composition of ρ-cymene in the extract was too low to be detected. There are some variations in the qualitative and quantitative compositions of *Nigella sativa* essential oil in different regions of the world. Many factors can influence the essential oil composition of the seeds including genetic and phenologic stage, as well as environment effects and extraction methods (Reineccius 1994; Omidbaigi 1997; Gora *et al*. 2002). The major bioactive compound: Thymoquinone was successfully isolated and characterized from $SCCO_2$ extract.

3.5. Conclusion

As a whole, forty seven different compounds were identified in supercritical CO_2 ($SCCO_2$-1 & 2) and HD $SCCO_2$ (hydrodistillation of $SCCO_2$-2 to obtain essential oil) extracts of which the occurrence of 16 volatile compounds were reported for the first time in *Nigella sativa* seeds. HD $SCCO_2$ gave an essential oil of better quality than the extracts obtained by $SCCO_2$ alone. This is a first report of its kind on the chemical composition of essential oil obtained by hydrodistillation of $SCCO_2$ extract of *Nigella sativa* seeds. The presence of pharmacologically active four major quinonic phenol compounds like thymoquinone (TQ), thymohydroquinone (THQ), thymol (THY) and dithymoquinone (DTQ) were confirmed to be present as valuable bioactive profiles of the $SCCO_2$ extracts. The major bioactive compound was isolated and characterized as thymoquinone. This study has clearly brought out the possibilities of obtaining higher percentage of volatiles such as thymoquinone through the $SCCO_2$ technology and proved its superiority to earlier extractions, findings and reports.

Hence, the characterized extracts and isolated major bioactive compound: Thymoquinone could further be subjected to application oriented studies like evaluation of antibacterial and antioxidant properties. This is in accordance with the objectives of the present work.

CHAPTER 4

Antibacterial properties of *Nigella sativa* L. seed extracts

CHAPTER 4

4.1 Introduction

One of the central themes of success in human therapeutics in the 20th century was the discovery and development of antibiotics and antibacterial agents, for the treatment of bacterial infections. A huge array of antibacterial agents has been introduced and antibiotics can be used effectively to treat major infectious diseases. However, the usage of antibiotics and antibacterial chemotherapeutics is becoming more and more restricted and complicated, as bacteria are capable of developing resistance to antibiotics soon after their introduction and most of the antibiotics have side effects. The problem of microbial resistance and degenerative diseases are growing and the outlook for the use of synthetic drugs without adverse effects for the future is still uncertain.

So far, 47 different compounds have been identified in the $SCCO_2$ (1 & 2) and HD $SCCO_2$ extracts of the *Nigella sativa* seeds. Of the compounds reported, 16 have been for the first time in the seeds. In order to evaluate, the bioactive molecules present in the $SCCO_2$ and conventional Soxtec™ extracts and isolated thymoquinone are focused on the evaluation of antibacterial properties against different food borne bacteria. Based on this evaluation, antibacterial principle is identified.

Food poisoning

Food borne illness is a major public health concern worldwide. Because the costs in terms of human illness and economic losses to individual companies and to the public health sector are immense. Several recent large food poisoning outbreaks in the UK, caused by *Salmonella,* have had, at their root, the purchase of (presumably) cheaper eggs from outside the UK, where there is no vaccination policy (Laurie *et al.* 2003).

'Emerging' pathogens are the subject of much research and discussion and they continue to present new challenges for identification and control. Changing consumer tastes and demands mean that some unlikely organisms may emerge over the recent decade, and it is the job of microbiologists to anticipate and control these new organisms through novel techniques. It is important to remember at less than 20 years ago, *Listeria monocytogenes* was almost an obscure species outside the veterinary field. But currently it again becomes a risk in that field. *Campylobacter* and *E. coli* have emerged a significant cause of food poisoning from the consumption of poultry and in ground beef respectively. In most cases the food poisoning involves gastroenteritis-vomiting and/or diarrhoea - but in the cases of botulism and listeriosis, the main symptoms are caused by effects on other ('extra-intestinal') parts of the body (Laurie *et al.* 2003).

Food borne bacterial pathogens

Bacillus cereus

Bacillus cereus gram-positive, endospore-forming, motile rod shaped bacterium with peritrichous flagellae belonging to the family Bacillaceae. It grows best aerobically as well as anaerobically. Sporulation readily occurs only in the presence of oxygen. It tends to grow in long chains. The cell width is ≥ 0.9 μm and it produces central to terminal ellipsoid or cylindrical spores that do not distend the sporangia. Under anaerobic conditions both growth and toxin production are reduced. *Bacillus cereus* is now recognized as a significant cause of food borne illness in humans. It is associated with 2 types of food borne illnesses, namely the "emetic" (vomiting) type and "diarrhoeal" responses caused by the formation of toxins (Doyle 1988a).

Staphylococcus aureus

Staphylococcus aureus Gram-positive, catalase-positive, oxidase negative coccus, belonging to the family Micrococcaceae, which is capable of producing heat-stable toxins (enterotoxins) in food. Cells form characteristic clumps resemble to bunch of grapes. It can grow either aerobically or anaerobically (Reed 1993; Sutherland and Varnam 2002; Bergdoll and Lee Wong 2005). It is generally caused by the ingestion of a toxin pre-formed in contaminated food. Although the illness produced is not considered to be particularly serious, it is quite common on a worldwide basis and occurs either sporadically or in significant outbreaks. *Staphylococcal* food intoxication is one of the most common food borne illnesses (Newsome 1988).

Escherichia coli

Enteropathogenic *Escherichia coli* are significant cause of diarrhea in developing countries, especially in localities with poor sanitation. The presence of *E. coli* in water or foods may indicate faecal contamination, although their consumption may not lead to any apparent ill effects on health. Certain serotypes of *E. coli,* may cause diarrhoeal disease, or more serious forms of illness. *E. coli* is facultative anaerobic, Gram negative non-spore-forming, catalase-positive, oxidase-negative, motile rods. It can be broken down into a number of serotypes, which produce their own particular toxin. *E. coli* is part of the normal flora of the intestinal tract of both man and warm-blooded animals. *E. coli* can be divided into several virulence groups, described as enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), diffusely adherent (DAEC), enteroaggregative (EAggEC) or vero cytotoxigenic (VTEC) (also described as Shiga-like toxin-producing, STEC). The last

group includes those serotypes described as enterohaemorrhagic (EHEC) (Frank 1988).

Yersinia enterocolitica

Yersiniosis, a form of gastroenteritis illness, is caused by the consumption of food containing viable pathogenic *Yersinia enterocolitica*, which has the ability to grow and proliferate at refrigerated temperatures (Doyle 1988b). *Yersinia enterocolitica* is Gram-negative, oxidase negative, catalase positive non-sporing rods (occasionally coccoid). Like other members of this family, *Yersinia* is a facultative anaerobe that ferments glucose. They are non-motile at 35-37°C, but motile at 22-25°C; however, some human pathogenic strains of serovar O:3 are non-motile at both temperatures (Nesbakeen 2006a, 2006b).

Medicinal plants

An alarming increase in bacterial pathogens resistant to a number of antimicrobial agents demands that a renewed effort be made to find out antibacterial agents effective against pathogenic bacteria resistant to the antibiotics that are currently in practice. Most of the synthetic drugs block receptor sites and hence attempts are being made to control the use of synthetic drugs and develop new drugs from natural resources like medicinal plants. One of the possible strategies towards this objective is the rational localization of bioactive phytochemicals. Medicinal plants are important therapeutic aids for various ailments and the use of those that are native to India in various traditional systems of medicine are awe inspiring. It is essential to work for newer drugs from medicinal plants with lesser or no toxicity. Traditional healers have long used various plant parts like roots, tubers, stems, leaves, flowers, fruits and seeds to prevent or cure infectious conditions. Many of these seeds

have been investigated scientifically for antimicrobial activity and a large number of plant products have been shown to inhibit growth of pathogenic bacteria.

Black cumin seeds

Pharmacologically active seeds of *Nigella sativa* L. are found in southern Europe, northern Africa and Asia Minor. The seeds are small, black and possess aromatic odor and taste. The black cumin seeds have been extensively investigated in recent years and used in folk medicine as a natural remedy for a number of diseases such as asthma, hypertension, diabetes, inflammation, cough, eczema, fever and gastrointestinal disturbances. Seed oil also has antipyretic, analgesic and antineoplastic activity (Ali and Blunden 2003). Thymoquinone, a major bioactive compound of *Nigella sativa* seeds, is a pharmacologically active quinone, which possesses several pharmacological properties including analgesic and anti-inflammatory actions (Abdel Fattah *et al*., 2000; Randhawa and Al Ghamdi, 2002).

Extraction of bioactive compounds

Selection of extraction technique is important to extract active substances or group of bioactive compounds of interest. Many of these bioactive compounds possess high volatility, thermo sensitivity and photo reactivity. Hence, the evaluation of the extraction process related to its efficiency to reach target compounds from a solid matrix is of considerable relevance.

Supercritical fluid extraction (SFE) of bioactive compounds from plant material is a promising field for the industrial application (Reverchon 1997), since it has certain advantages over steam-distillation and solvent extraction. Steam-distillation can lead to thermal degradation and partial hydrolysis of some essential oil compounds, while SFE can be performed at lower temperatures, thereby preserving the original extract composition and properties. Carbon dioxide is the most used

supercritical solvent in extractions of active constituents, especially for application in pharmaceutical, cosmetic and food industries. Carbon dioxide is non toxic and allows SFE at temperatures near room temperature and relatively low pressures (8-10 MPa). To suppress co-extraction of higher molecular weight constituents, Reverchon (1997) suggested performing SFE of essential oil at temperatures of 40-50°C and pressures below 10 MPa.

The present study was carried out on the antibacterial properties of *Nigella sativa* seed extracts, obtained by $SCCO_2$ and conventional Soxtec™ extractions against different food borne bacteria. Further, the isolated thymoquinone was evaluated for its MIC and MBC determinations. However only a limited data is available so far regarding antibacterial properties of isolated thymoquinone from $SCCO_2$ extracts of *Nigella sativa* seed against different food borne bacterial pathogens. The present study was therefore focused to evaluate on these aspect of *Nigella sativa*.

4.2. Materials and methods

Materials and reagents

Nigella sativa seeds were obtained from Supreem Pharmaceuticals Mysore Pvt. Ltd., India. Cleaned seeds were pulverized into a fine powder by an IKA-Analytical Mill A10 (Janke & Kunkel GmbH & Co. KG, Germany). Analytical grade solvents from Merck Co, India; Propylene glycol from SISCO, Research Laboratories Pvt. Ltd., India; Nutrient agar (NA), brain heart infusion (BHI) agar and Mueller–Hinton agar (MHA) media, Ciprofloxacin antibiotic, D-glucose, DMSO, phenol red indicator and sterile antimicrobial susceptibility testing disks (6.0 mm dia) were obtained from HiMedia Laboratories Ltd., Mumbai, India. Food grade CO_2 cylinder (of 99.9% purity) was obtained from Kiran Corporation, Mysore, India.

Test bacteria

Various food borne pathogenic bacteria like *Bacillus cereus* F 4810 (Public Health Laboratory, London, UK), *Staphylococcus aureus* FRI 722 (Public Health Laboratory, The Netherlands), *Escherichia coli* MTCC 108 and *Yersinia enterocolitica* MTCC 859 (Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India) were employed in this present study.

$SCCO_2$ extraction

$SCCO_2$ extraction was carried out in high pressure extractor. Extractions were carried out on 1 kg of the pulverized black cumin seeds at 40°C and 50°C at 120 and 280 bar pressures. CO_2 mass flow rate of 3.30-8.05 $\times$ 10^{-4} kg/s was employed to obtain $SCCO_2$-1 (120 bar, 40°C) and $SCCO_2$-2 (280 bar, 50°C) extracts. Extracts were then stored at 4°C in dark until analysis. $SCCO_2$ extraction was carried out as described earlier in Materials and Methods section (sub heading as Extraction using Supercritical CO_2) in Chapter 2.

Soxtec^{TM} extraction

Powdered seed material was extracted in hexane, ethyl acetate, methanol and methanol: water (70:30 v/v) solvents in a Soxtec^{TM} apparatus. After the extraction, the extracts were concentrated by evaporation of solvents using Buchi evaporator and further subjected to stream of nitrogen. The obtained residue/solvent free extract/oil was collected and used for further studies. Soxtec^{TM} extraction was performed as described in Materials and Methods section of Chapter 2.

The extractive yield of both $SCCO_2$ and Soxtec^{TM} extracts on dry weight basis of seeds were calculated and mentioned elsewhere in Chapter 2.

Preparation of extracts

The known quantities of solvent free (Soxtec^{TM}) and $SCCO_2$ extracts were dissolved in propylene glycol for evaluation of antibacterial properties.

Preparation of culture media

Mueller–Hinton agar (MHA), nutrient agar (NA) and brain heart infusion (BHI) agar media and respective broths were prepared and autoclaved at 121°C, 15 psi for 15 min.

Preliminary screening of antibacterial activity

Antibacterial activities of *Nigella sativa* seed extracts were preliminarily screened by the disk diffusion technique (Bauer *et al.* 1966; Alzoreky and Nakahara 2003). The Mueller–Hinton agar (MHA) plates containing an inoculum size of 10^6 colony-forming units (CFU)/mL of broth were spread on the solid plates with an L-shaped glass rod. Then disks (of 6.0-mm dia) impregnated with 25 µL of each extracts at a concentration of 10.0 mg/mL were placed on the inoculated plates. Similarly, each plate carried a blank disk by adding DMSO solvent alone in the center to serve as a negative control and for comparative purposes whereas a blank disk impregnated

with 25 µL of ciprofloxacin antibiotic at a concentration of 2 mg/mL, was used as positive control. All the plates were incubated at 37°C for 20 to 24 h. After the incubation period, the sensitivity of the bacterial pathogens to the extracts was determined by measuring the size of growth inhibitory zones (including the diameter of disk) on the agar surface around the disks.

Growth inhibition assay

Growth inhibition assay of *Nigella sativa* seed extracts against different bacteria were tested by agar dilution technique (Negi *et al*. 1999). For this *Yersinia enterocolitica* was grown in BHI agar and the remaining test bacteria were grown in nutrient agar at 37°C. Each bacterial strain was transferred from stored slants at 4–5°C to 10 ml of broth and incubated overnight at 37°C. A preculture was prepared by transferring 1 ml of culture to 9 ml of broth and was cultivated for 48 h. The cells were harvested by centrifugation (1200g, 5 min), washed and suspended in sterile saline under aseptic conditions.

To flasks containing 20 ml of melted nutrient agar/BHI agar, different concentrations (50 to 600 ppm) of respective extracts in propylene glycol were added. An equivalent amount of propylene glycol was used as control. One hundred µl (about 10^3 CFU/ml) of each bacterium to be tested was inoculated into the flasks under aseptic conditions. The media were then poured into sterile petri dishes, in triplicate, and incubated at 37°C for 20-24 h. After the incubation period, the colonies were enumerated and were used to find out the growth inhibition by using the following formula:

$$Inhibition\,(\%) = (1 - T/C) \times 100$$

Where T is cfu/ml of extract and C is cfu/ml of control.

The lowest concentration of the extracts capable of inhibiting the complete growth of the bacteria being tested was reported as minimum inhibitory concentration (MIC) values.

Antibacterial activities of thymoquinone

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of isolated thymoquinone was determined in nutrient broth containing 0.05% phenol red and supplemented with 10% glucose (NBGP) by microdilution technique (Zgoda and Porter 2001). Isolated compound (thymoquinone) and ciprofloxacin antibiotic were initially dissolved in DMSO individually and the solution obtained was added to NBGP to a final concentration of 5000 µg/ml. This was serially diluted by two fold, to obtain concentrations ranging from 5000µg to 1.22µg/ml. 100 µl of each concentration was added to a well (96-well microplate) containing 95 µl of NBGP and 5µl of inoculum, the appropriate inoculum size for standard MIC is 2×10^4 to 10^5 CFU/ml. The final concentration of DMSO in the well was less than 1%. The negative control well consisted of 195µl of NBGP and 5µl of the inoculum. The plates were covered with a sterile plate sealer, then agitated to mix the content of the wells using a plate shaker and incubated at 37°C for 24 h. The assay was repeated thrice and bacterial growth was determined by observing the change of color in the wells (red when there is no growth and yellow when there is growth). The lowest concentration showing no color change in the well was taken as the MIC.

Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) was determined according to the microdilution technique (Zgoda and Porter 2001). A volume of 20 ml of agar was dispersed on petridish and allowed to solidify. A portion of the liquid (5µl) from each well (96-well microplate) that showed no change in color was plated on agar plate and incubated at 37°C for 24 h. The lowest concentrations that yielded no growth after this sub-culture were recorded as the MBC.

Statistical analysis

All the experiments were performed in triplicate and the data were calculated as means ± SD. A probability value of $P < 0.05$ was considered significant.

4.3. Results

In the preliminary screening of antibacterial activity, using disk diffusion technique, $SCCO_2$ extracts have shown effective inhibition zones greater than 15 mm diameter in size than conventional solvent extracts obtained by Soxtec^{TM} system at a concentration of 250 µg/disc. The $SCCO_2$-1 (120 bar, 40^{o}C) extract from the seeds of *Nigella sativa* has shown largest inhibition zones (>15 mm diameter) against almost all the tested organisms except *Yersinia enterocolitica*. But smaller inhibition zones (< 10 mm diameter) were detected in Soxtec^{TM} extracts except ethyl acetate extract which has shown larger inhibition zones (>10 mm diameter) against Gram-positive bacteria and smaller inhibition zones (<10 mm diameter) against Gram-negative bacteria. Ciprofloxacin antibiotic was used as positive control which also shown inhibition zones greater than 15 mm diameter in size against all the tested bacteria except *Yersinia enterocolitica* at a concentration of 50 µg/disc. All the extracts were found to be active against Gram-positive than Gram-negative bacteria. All inhibition zones corresponding to test bacteria are presented in Table 4.1. The antibacterial evaluation tests indicate that the higher efficiency of supercritical CO_2 extracts compared with conventional solvent extracts of Soxtec^{TM} system against the test bacteria.

Table 4.1. Preliminary screening of *Nigella sativa* L. seed extracts for antibacterial activity by disk diffusion technique.

Extracts	Zone of inhibition (mm)			
	Bacillus cereus	*Staphylococcus aureus*	*Escherichia coli*	*Yersinia enterocolitica*
$SCCO_2$-1 (120 bar, 40°C)	+ + +	+ + +	+ + +	+ +
$SCCO_2$-2 (280 bar, 50°C)	+ +	+ +	+ +	+
Ethyl acetate	+ +	+ +	+	+
Methanol	+	+	+	+
Hexane	+	+	+	—
Methanol: water (70:30 v/v)	+	+	+	—
Ciprofloxacin	+ + +	+ + +	+ + +	+ +
DMSO	—	—	—	—

Key: —, no inhibition zone; +, inhibition zones of < 10 mm diameter; + +, inhibition zones of > 10 mm diameter; + + +, inhibition zones of > 15 mm diameter. Sterile disks (6.0-mm diameter) impregnated with 250 µg of extracts and 50 µg of Ciprofloxacin antibiotic was used as positive control and 25 µl of DMSO was used as negative control.

The investigation also reveals that the $SCCO_2$ extracts showed an effective bacterial growth inhibition than the extracts of Soxtec™ system. The $SCCO_2$-1 (120 bar, 40°C) extract has shown effective growth inhibition on all the organisms tested, which was followed by $SCCO_2$-2 and then by ethyl acetate extracts. But their efficiency to inhibit the growth was varied from one organism to another. *Bacillus cereus*, *Staphylococcus aureus* and *Escherichia coli* showed maximum growth inhibition to both $SCCO_2$ and the extracts of Soxtec™ system whereas *Yersinia enterocolitica* showed maximum growth inhibition to $SCCO_2$ and ethyl acetate extracts (Figs 4.1—4.4). These observations were also reflected in the MIC of these extracts for different bacteria. MIC of the $SCCO_2$-1 (120bar, 40°C) extract for tested bacteria was the lowest at 100-350 ppm followed by $SCCO_2$-2 (280 bar, 50°C) and ethyl acetate extracts. MIC was also varied from one organism to another and all the organisms were more sensitive to $SCCO_2$ extracts except *Yersinia enterocolitica*. *Bacillus cereus* and *Staphylococcus aureus* were found to be more susceptible to $SCCO_2$ extracts than the conventional solvent extracts of Soxtec™ system. *Escherichia coli* was equally sensitive to both the extracts of $SCCO_2$ and Soxtec™ system. *Yersinia enterocolitica* was the most resistant bacterium to all the tested extracts as inferred from higher MIC values observed for it (Table 4.2).

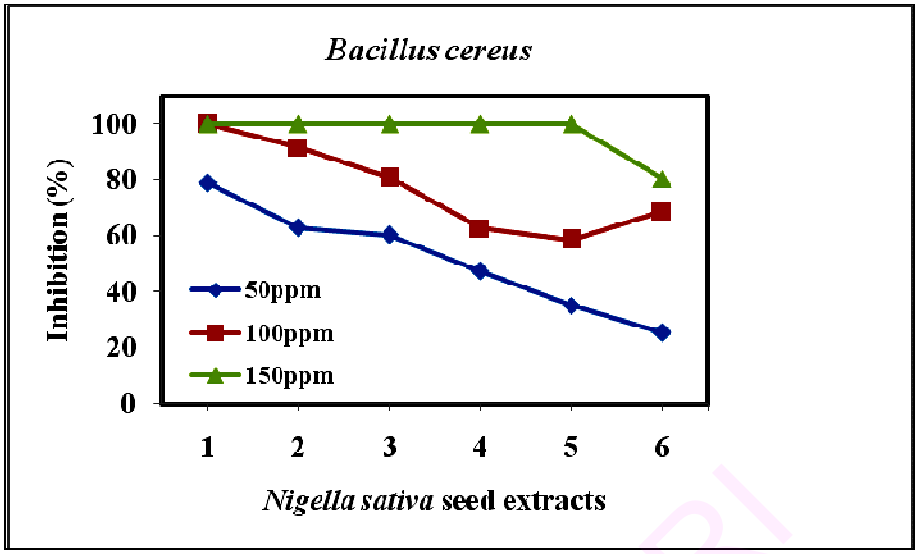


Fig. 4.1. Inhibition of *Bacillus cereus* growth by *Nigella sativa* L. seed extracts: 1. $SCCO_2$-1 (120 bar, 40°C), 2. $SCCO_2$-2 (280 bar, 50°C), 3. Ethyl acetate, 4. Methanol, 5. Hexane and 6. Methanol: Water (70:30 v/v) extracts. (SD values varied from 0-1.70).

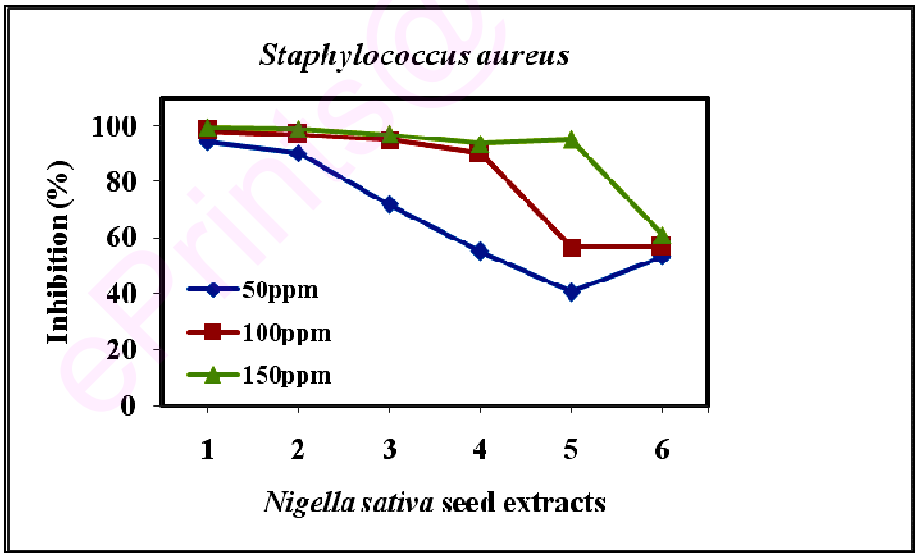


Fig. 4.2. Inhibition of *Staphylococcus aureus* growth by *Nigella sativa* L. seed extracts: 1. $SCCO_2$-1 (120 bar, 40°C), 2. $SCCO_2$-2 (280 bar, 50°C), 3. Ethyl acetate, 4. Methanol, 5. Hexane and 6. Methanol: Water (70:30 v/v) extracts. (SD values varied from 0.39-2.03).

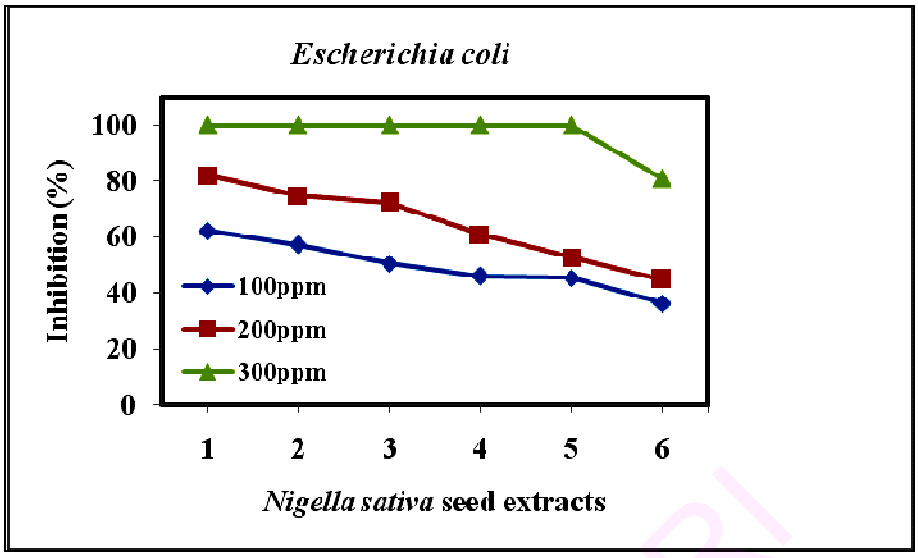


Fig. 4.3. Inhibition of *Escherichia coli* growth by *Nigella sativa* L. seed extracts: 1. $SCCO_2$-1 (120 bar, 40°C), 2. $SCCO_2$-2 (280 bar, 50°C), 3. Ethyl acetate, 4. Methanol, 5. Hexane and 6. Methanol: Water (70:30 v/v) extracts. (SD values varied from 0-1.68).

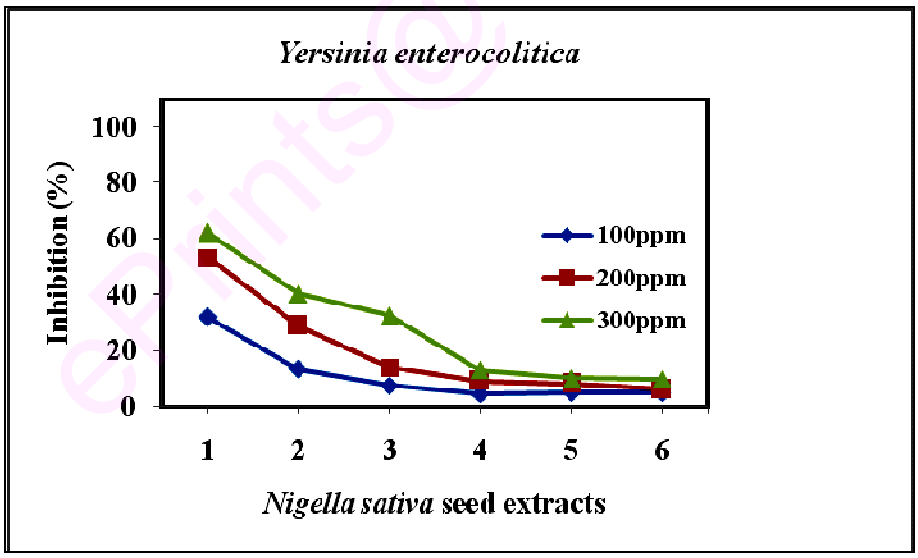


Fig. 4.4. Inhibition of *Yersinia enterocolitica* growth by *Nigella sativa* L. seed extracts: 1. $SCCO_2$-1 (120 bar, 40°C), 2. $SCCO_2$-2 (280 bar, 50°C), 3. Ethyl acetate, 4. Methanol, 5. Hexane and 6. Methanol: Water (70:30v/v) extracts. (SD values varied from 0.10-1.42).

Table 4.2. Minimum inhibitory concentration (MIC) of black cumin seed extracts.

Extracts	MIC[a] (ppm)			
	Bacillus cereus	*Staphylococcus aureus*	*Escherichia coli*	*Yersinia enterocolitica*
$SCCO_2$-1 (120 bar, 40°C)	100	175	300	350
$SCCO_2$-2 (280 bar, 50°C)	150	175	300	450
Ethyl acetate	150	200	300	500
Methanol	150	200	300	500
Hexane	150	225	300	550
Methanol: water (70:30 v/v)	200	250	350	550

[a]MIC is defined as the lowest concentration of the extract capable of inhibiting the complete growth of the bacterium.

Using the calibration curve, the quantification of thymoquinone (TQ) was achieved at 254 nm ranging from 0.6 to 12.3% in all the obtained extracts. $SCCO_2$-1 extract had higher thymoquinone content of 12.3%. The extract yields were calculated based on the dry weight of the seed material and it ranged from 10.6 to 42.7% whereas $SCCO_2$-1 (120 bar, 40°C) extract had lower extract yield of 16.2% (Tables 2.3, 2.4 and 2.7 of Chapter 2). Based on this higher TQ content of $SCCO_2$-1 extract which resulted effective antibacterial activity among the six extracts studied.

MIC and MBC determinations of thymoquinone

Isolated thymoquinone was subjected to Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determinations by microdilution technique. It was tested at different concentrations for antibacterial activity, the extent of their inhibitory activities against the test bacteria could be well understood by comparing the MIC and MBC values obtained. The MIC and MBC values of isolated thymoquinone against different bacteria are presented in Table 4.3.

Table 4.3. MIC and MBC values of isolated thymoquinone against different food borne pathogenic bacteria.

Bacterial pathogens	MIC[a] (µg/ml)		MBC[b] (µg/ml)	
	Thymoquinone	Standard[c]	Thymoquinone	Standard[c]
Bacillus cereus	19.5	4.8	39.0	9.7
Staphylococcus aureus	39.0	9.7	39.0	19.5
Escherichia coli	39.0	9.7	78.1	19.5
Yersinia enterocolitica	78.1	19.5	156.2	39.0

[a]MIC: Minimum Inhibitory Concentration; [b]MBC: Minimum Bactericidal Concentration; [c]Ciprofloxacin antibiotic.

The results revealed that *Bacillus cereus* (MIC: 19.5 µg/ml) was most sensitive to thymoquinone whereas *Staphylococcus aureus* (MIC: 39 µg/ml) and *Escherichia coli* (MIC: 39 µg/ml) showed equal sensitivity whereas *Yersinia enterocolitica* (MIC: 78.1 µg/ml) showed resistance to thymoquinone, among all those tested bacteria. All the tested bacterial MIC ranged from 19.5 to 78.1 µg/ml. The lower MBC values of 39 µg/ml against *Bacillus cereus* and *Staphylococcus aureus* whereas the values of 78.1 µg/ml against *Escherichia coli* and 156.2 µg/ml against *Yersinia enterocolitica* were recorded for the isolated thymoquinone. A range from 39 to 156.2 µg/ml was observed as MBC for all tested bacteria. MIC and MBC values for Ciprofloxacin antibiotic was ranged from 4.8 to 19.5 µg/ml and 9.7 to 39 µg/ml, respectively. The isolated TQ has significant MIC and MBC values against food borne bacterial pathogens.

4.4. Discussion

In this present investigation, $SCCO_2$ extracts have shown largest inhibition zones than conventional solvent extracts using disk diffusion technique against almost all the tested organisms except *Yersinia enterocolitica*. Both $SCCO_2$ and conventional Soxtec^{TM} extracts were found to be active against Gram-positive than Gram-negative bacteria. Although the disk diffusion method is sensitive to detect microbial growth, it has a qualitative character and should not be recommended to quantify the antimicrobial activity of a substance based on the size of the inhibition zone formed during the analyses (Rios *et al.* 1988). In this study, $SCCO_2$-1 extract had higher thymoquinone content (of 12.3%) which is in agreement with similar values reported earlier (Erkan *et al*. 2008).

The $SCCO_2$ extracts have also shown effective bacterial growth inhibition against the organisms tested than the extracts of Soxtec^{TM} system using agar dilution technique. *Yersinia enterocolitica* showed maximum growth inhibition to $SCCO_2$ and ethyl acetate extracts only. These observations were also reflected in the MIC values of these extracts for different bacteria. MIC also varied from one organism to another and the tested organisms were more sensitive to $SCCO_2$ extracts except *Yersinia enterocolitica*. It is the most resistant bacterium to all the tested extracts as inferred from higher MIC values observed for it. This antibacterial evaluation tests indicate higher efficiency of supercritical CO_2 extracts than conventional solvent extracts (of Soxtec^{TM} system) against the test bacteria. The reason might be the no solvent interference and also the higher TQ content in the $SCCO_2$ extracts.

In this study, MICs ranged from 19.5 to 78.1 µg/ml and MBCs ranged from 39 to 156.2 µg/ml were observed for isolated thymoquinone (TQ) against the tested bacteria. But *Yersinia enterocolitica* showed resistance to isolated thymoquinone

(MIC: 78.1 µg/ml; MBC: 156.2 µg/ml). In this study, all the extracts and isolated TQ were found to be active against Gram-positive than Gram-negative bacteria. The higher resistance of Gram-negative bacteria to external agents has earlier been documented and it is attributed to the presence of lipopolysaccharides in their outer membranes, which make them inherently resistant to antibiotics, detergents and hydrophilic dyes (Nikaido and Vaara 1985). Results of the present study are consistent with the literature data reported earlier (Alhaj *et al*. 2008; Shah and Sen ray 2003; De *et al.* 1999; Agarwal *et al*. 1979).

Previous studies were reported on 8 MRSA (methicillin resistant *Staphylococcus aureus*) and 4 MSSA (methicillin sensitive *Staphylococcus aureus*) strains and evaluated them against aqueous and ethanolic extracts of *N. sativa*. MIC was found to be 0.04 mg/ml for both MRSA and MSSA (Dadgar *et al.* 2006). The reason may be the difference in the methods adopted for the extract preparation and performance of MIC studies. It has also been found that all tested strains of MRSA were sensitive to *N. sativa* extract at a concentration of 4 mg/disc, while the extract had an MIC range of 0.2–0.5 mg/ml. The results of disk diffusion assay demonstrated that MRSA strains were completely inhibited at 4 mg/disc was considered to be significant. However a concentration of 0.5 mg per disk failed to inhibit any of these strains (Hannan *et al.* 2008).

Earlier studies on the antimicrobial activity of *N. sativa* seed essential oil obtained by $SCCO_2$ extraction were investigated against Gram positive and Gram negative strains. Filter paper disks impregnated with varying concentrations of extract were tested by the disk diffusion method against various bacteria. The inhibition zones of the Mueller Hinton agar in different extract concentration showed that at 25 mg 20 μL^{-1}, 50 mg 20 μL^{-1} and 100 mg 20 μL^{-1}, the inhibition zones were increased

accordingly in *S. aureus*. However, *N. sativa* was found to be inactive against *E. coli* and *K. pneumoniae*) (Alhaj *et al.* 2008). The present results have good agreement in inhibition zones obtained for *S. aureus* and *E. coli*.

Previous reports on two main components of black seed essential oil, thymoquinone (TQ) and thymohydroquinone (THQ) were investigated for their antibacterial activity (Halawani 2009). Both TQ and THQ exerted antibacterial activity against both Gram-positive and Gram-negative bacteria regardless of their susceptibility to antibiotics. *S. aureus*, was highly susceptible to TQ. This study demonstrated that both TQ and THQ have antibacterial activity (Halawani 2009). TQ exhibited a significant bactericidal activity against the majority of the tested human pathogenic bacteria especially Gram positive cocci (*Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* CIP 106510) (Chaieb *et al.* 2011). TQ present in volatile oil obtained from the crude extract exhibited remarkable growth inhibition on various strains of bacteria (Kahsai 2002). In this present work also, TQ exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria. Even though, Gram-negative bacteria were less susceptible to TQ especially *Yersinia enterocolitica,* which also reflected in MIC and MBC values.

Generally, the quantity of the extract/ isolated compound required to inhibit bacteria was low in agar and microdilution techniques as compared to the disk diffusion technique. This is likely to be acceptable because of the differences in methodology of the three techniques. In agar dilution technique the extract is directly incorporated into the medium whereas in microdilution technique small quantity of isolated compound (thymoquinone) is placed in microplate wells therefore the bacteria are brought into direct contact with the isolated compound rather than relying on diffusion of constituents through the disc. The concentration of the ingredients is

always higher in agar dilution technique whereas at microlitre levels in microdilution technique next to the disk and here decreases gradually. Since bacteria give rise to a new generation every 18–20 minutes the inoculated plates containing disks at 37°C help bacteria to grow while components of the extract need some time to diffuse through the disc and exert their effects. These factors point out the lack of sensitivity of the disk diffusion technique therefore it is recommended that interpretation of results of diffusion tests must be based on the comparison between microdilution, agar dilution and diffusion methods comprehensively. These comparisons are useful in establishing reference standards. Such comparisons were employed in this study.

4.5. Conclusion

The investigation has revealed that the $SCCO_2$ extracts have an effective bacterial growth inhibition than the solvent based Soxtec™ extracts. The maximum content of TQ is possible only in $SCCO_2$ extraction while exhibiting a direct correlation with the antibacterial activity of the extract. The present study thus established that the $SCCO_2$ extracts of *Nigella sativa* L. seeds were active against almost all of the tested food borne bacteria, due to the higher TQ content. Also indicated through the evaluation study is that the antibiotic resistance of food borne bacterial pathogens does not interfere in the antimicrobial action of the *Nigella sativa* L. seed extracts and thymoquinone. The isolated TQ showed significant MIC and MBC values against food borne bacteria, which confirmed it could be the antibacterial principle for *Nigella sativa* L. seed. Hence, this antibacterial principle: TQ appears to be promising as a potential natural antibacterial agent and its broad spectrum antibacterial activities are comparable to Ciprofloxacin antibiotic and may be with other antibiotics also, which are used currently.

The *Nigella sativa* L. seed extracts and the isolated thymoquinone could be taken up for their antioxidant properties as a next step of evaluation, in accordance with the objectives of the present research.

CHAPTER 5

Antioxidant properties

of

Nigella sativa L. seed extracts

CHAPTER 5

5.1. Introduction

The *Nigella sativa* L. seed extracts (both conventional and supercritical carbon dioxide) were evaluated for antibacterial properties along with the isolated thymoquinone. Here a similar evaluation for the antioxidant properties of the same extracts and thymoquinone are conducted as per the objectives of the research.

Interest in newer sources of edible oils has recently grown to a great extent due to the demands created by the growing global population. Hence, nonconventional oilseeds are being considered for food grade applications as their phytochemicals have unique chemical properties and may augment the supply of edible oils. Among the various seed oils, black cumin (*Nigella sativa* L.) is of particular interest because it may be utilized for the production of formulations containing phytochemicals with significant antioxidant properties and health benefits. Very little information, however, is available on the phytochemicals present in the *Nigella sativa* seed oil, that are responsible for the antioxidant properties. The seed oil has been a part of a supplemental diet in many parts of the world and its consumption is also becoming increasingly popular in the nonproducer countries. Information on the phytochemicals in that oil is limited, yet these phytochemicals especially, thymoquinone (TQ) may bring nutraceutical and functional benefits to food systems.

Free radicals

Atoms contain a nucleus, and electrons move around the nucleus, usually in pairs. A free radical is any atom or molecule that contains one or more unpaired electrons. The unpaired electrons alter the chemical reactivity of an atom or molecule, usually making it more reactive than the corresponding non-radical. However, the actual chemical reactivity of radicals varies enormously. The hydrogen radical (H•,

the same as a hydrogen atom), which contains 1 proton and 1 electron (therefore unpaired); is the simplest free radical. Free radical chain reactions are often initiated by removal of H• from other molecules (eg, during lipid peroxidation). A superscripted dot is used to denote free radicals. When oxygen is metabolized, it creates 'free radicals', which steal electrons from other molecules. Free radicals play a crucial role in the immune system; they float through the veins and attack foreign invaders. They promote beneficial oxidation that produces energy and kill bacterial invaders. The body can cope with some free radicals and needs them to function effectively. However, an overload of free radicals has been linked to certain diseases. A poor diet, stress, cigarette smoking, alcohol, sunlight, ultraviolet (UV) radiation, pollution and other factors can accelerate oxidation and the situation known as "oxidative stress" is believed to play a leading role in certain diseases and age related changes. The body cannot turn air and food into chemical energy without a chain reaction of free radicals. Once formed, these highly reactive radicals can start a chain reaction, like dominoes. Their chief danger comes from the damage they can do when they react with important cellular components such as DNA, or the cell membrane. Cells may function poorly or die as a result. Some oxygen-derived free radicals include super oxide, hydroxyl (OH), hydroperoxyl (HOO), peroxyl (ROO) and alkoxyl (RO) radicals. Other common reactive oxygen species (ROS) include nitric oxide (NO), and the peroxynitrite anion (ONOO) (Halliwell and Gutteridge 1989).

Antioxidants

Antioxidants are substances that can inhibit the generation of free radicals, which initiate lipid oxidation or reduce the number of free radicals in a system and eventually prevent or delay the lipid oxidation reaction. Lipid oxidation causes serious food quality deterioration during the storage of foods containing lipids. The

oxidation of lipids produces undesirable rancid odors and oxidation products and thereby decreases the nutritional quality of foods. Antioxidants can quench and terminate free radicals without transforming themselves in to new free radicals in the system. Antioxidants are generally categorized in two classes. They are *synthetic or artificial antioxidants*, (which are produced by artificial synthesis reactions) and *natural antioxidants*, (which are produced, accumulated and excreted in a biological system). The most common synthetic antioxidants used in the food industry are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ) and propyl gallate (PG). All of them are either monohydric or polyhydric phenols with other substitutes on the phenol ring. The hydroxyl groups on the phenol ring contribute to the antioxidant function of those chemicals through by donating electrons to annihilate free radicals in a system. Most antioxidants from natural sources also have monohydric or polyhydric phenols in their chemical structures, which are called phenolics (Madhavi *et al.* 1996).

The usage of synthetic antioxidants is limited and restricted because of food safety concerns. Even though a small amount of these artificial chemicals are used, they are still a concern because of potential harmful health problems from long term consumption (Kotsonis *et al*. 2001) and could form promoting agents that target liver, lung and stomach tissues to alter their gene expression (Pitot and Dragon 2001). Synthetic antioxidants also show low solubility and moderate antioxidant activity (Barlow 1990; Branen 1975). Currently available synthetic antioxidants like BHA, BHT, TBHQ and gallic acid esters have proven ill effects. The most commonly used synthetic antioxidants are BHA, BHT and TBHQ, which are applied in fat and oily foods to prevent oxidative deterioration (Loliger 1991). However, BHA and BHT were found to be anticarcinogenic as well as carcinogenic in experimental animals.

Originally, BHA appeared to have tumor-initiating as well as tumor-promoting action. Recently, it has been established that tumor formation appears to involve only tumor promotion caused by BHA and BHT (Botterweck *et al.* 2000). Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring phenolic antioxidants. This is to minimize or retard oxidative deterioration in food and to improve the health-related functional values of the food (Botsoglou *et al.* 2002; Shetty 1997).

Antioxidant properties of phytochemicals

Many studies suggested that some phytochemicals in plants play an important role in preventing diseases. The phytochemicals are usually phenolics with monohydric or polyhydric phenols in their structures. They have antioxidant activity of inhibiting fatty acid and cholesterol oxidation and reduce the harmful lipid oxidation products in foods and the human system (Steinmetz and Potter 1996; Harborne and Williams 2000; Truswell 2002; Delgado-Vargas and Paredes-Lopez 2003; Boyer and Liu 2004). Plants have excellent antioxidant properties mainly attributable to the phenolic compounds contained in them. The phenolic antioxidants are products of secondary metabolism in plants and are good sources of natural antioxidants in human diets. Aromatic plants such as herbs and spices are especially rich in their phenolic content, and have been widely used to extend the shelf life of foods (Botsoglou *et al.* 2002; Adam *et al.* 1998) and in traditional medicine as a treatment for many diseases (Khanna *et al.* 1993). The antioxidant activity of phenolic compounds in plants is mainly due to their redox properties and chemical structure, which can play an important role in neutralizing free radicals, chelating transitional metals and quenching singlet and triplet oxygen, by delocalization or decomposing peroxides (Zheng and Wang 2001; Pizzale *et al.* 2002). The

antioxidants from natural sources are usually considered as GRAS-generally recognized as safe. With this advantage, consumers and food developers prefer using natural antioxidants to replace synthetic antioxidants in food products. Foods labeled 'all natural' or 'no artificial' are becoming quite common in the market nowadays.

Pharmacological properties of *Nigella sativa* L. seed

In recent times a considerable research interest has been devoted worldwide to investigate the *Nigella sativa* seeds for their pharmacological properties. It has been traditionally used in the Indian subcontinent, Arabian countries and Europe for medicinal purposes (Ali and Blunden 2003). In particular the seed oil has been evaluated and shown to have antihypertensive (El Tahir *et al.* 1993; Mahfouz *et al.* 1962), anthelmintic (Agarwal *et al*. 1979), analgesic (Khanna *et al*. 1993), anti-inflammatory (Houghton *et al*. 1995) and immunopotentiating activities (Medenica *et al*. 1993; Kandil *et al*. 1994). Many of these activities have been attributed to quinone compounds of thymoquinone, dithymoquinone, thymohydroquinone and thymol in the seed (Ghosheh *et al*. 1999). Radad *et al*. 2009 worked on the primary dopaminergic cultures from mouse mesencephala to investigate the neuroprotective effects of thymoquinone (TQ) against MPP (+) and rotenone toxicities and reported that the potential of TQ to protect primary dopaminergic neurons against MPP (+) and rotenone relevant to Parkinson's disease. Highly active anti-retroviral therapy (HAART) regimen has considerably reduced the mortality rate in HIV-1 positive patients. The inclusion of HIV-1 protease inhibitors in HAART has been linked to the induction of insulin resistance syndrome. Significantly increased reactive oxygen species (ROS) generation and suppressed cytosolic, but not mitochondrial superoxide dismutase (SOD) levels. Treatment with TQ along with nelfinavir in HIV-1 positive patients significantly inhibited the effect of nelfinavir on augmented ROS production

and suppressed SOD levels. Both TQ and oil exposure increased glucose stimulated insulin secretion and ameliorated the suppressive effect of nelfinavir. These findings suggest that TQ may be used as a potential therapeutic agent to normalize the dysregulated insulin production observed in HAART treated patients (Chandra *et al*. 2009). Treatment with anticancer drugs such as cyclophosphamide (CTX) is associated with significant toxicity due to over-production of ROS, resulting in increased levels of oxidative stress. *Nigella sativa* oil (NSO) or its active ingredient TQ along with CTX, reduced the induced toxicity in male albino rats. Treatment with NSO or TQ induced significant reduction in overall toxicity. These results suggest that administration of NSO or TQ can lower CTX-induced toxicity indicating a potential clinical application for these agents (Alenzi *et al*. 2010).

Extraction of phytochemicals

CO_2 is an ideal solvent for the extraction of natural products. Supercritical carbon dioxide ($SCCO_2$) is an environment friendly solvent-free extraction method with low oxidative and thermal impacts offering the possibility of recovering intact natural compounds with minimal alteration of the active ingredients for preserving their curative properties (Moyler and Heath 1988; Reverchon and Senatore 1992). Supercritical fluid extraction helps in the extraction of volatile, thermo sensitive and photo reactive phytochemical like thymoquinone of a wide range of polarities.

The present study is carried out with the following objectives. To evaluate the *Nigella sativa* seed extracts (obtained by $SCCO_2$ and conventional Soxtec^{TM} extractions) and isolated thymoquinone for antioxidant properties along with determination of total phenolic content. To the best of our knowledge, antioxidant properties of thymoquinone isolated from $SCCO_2$ extracts of *Nigella sativa* seed have not been studied so far.

5.2. Materials and methods

Materials and reagents

Seeds of *Nigella sativa* were obtained from the Supreem Pharmaceuticals Mysore Pvt. Ltd., India. Impurities were separated from the seeds and the cleaned seeds were ground with an IKA-Analytical Mill A10 (Janke & Kunkel GmbH & Co. KG, Germany). Butylated hydroxyanisole (BHA), 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2, 4, 6, tripyridyl-s-triazine (TPTZ), trolox (6-hydroxy- 2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), gallic acid and α-tocopherol (Sigma-Aldrich, USA), Folin-Ciocalteau reagent (Loba Chemicals, India), Sodium acetate, sodium carbonate and ferric chloride (Rankem Fine Chemicals, India), Glacial acetic acid and hydrochloric acid (SD Fine-Chem Limited, India), Analytical grade solvents (Merck Co, India) and food grade CO_2 cylinder of 99.9% purity (Kiran Corporation, Mysore, India) were all used.

Extraction of *Nigella sativa* seeds

Supercritical CO_2 extractions were carried out in a high-pressure extractor (Nova Swiss Werke AG, model Ex 1000-1.4-1.2 type, Switzerland) as described in Materials and Methods section of Chapter 2. The CO_2 mass flow rate was maintained at 3.30-8.05 $\times$ 10^{-4} kg/s to obtain $SCCO_2$-1 (120 bar, 40°C) and $SCCO_2$-2 (280 bar, 50°C) extracts. Yields of extracts were monitored by the weight of the extract recovered and stored at 4°C in dark until further analysis.

The powdered seed material was extracted individually in 120 ml of hexane, ethyl acetate, methanol and methanol: water (70:30 v/v) solvents using a Soxtec™ apparatus (Model: Soxtec™ system HT2 1045 Extraction unit, Foss Tecator, Sweden). The obtained extracts were subjected to de solventing and ethanol was removed by a stream of nitrogen. The solvent free extract (oil) then obtained was used

for further studies. The extraction was carried out similar as described in Materials and Methods section in Chapter 2.

Preparation of extracts and thymoquinone

The known quantities of $SCCO_2$ and solvent free (Soxtec^{TM}) extracts and isolated thymoquinone were dissolved in pure ethanol (99%) for antioxidant studies.

Determination of total phenolic content (TPC)

The total phenolic content (TPC) of *Nigella sativa* seed extracts was determined by the method of Swain and Hillis (1959). 100 µL of extracts (500 µg in ethanol), 2450 µL of double distilled water and 150 µL of 0.25 N Folin- Ciocalteau reagent were combined in a test tube and then mixed well. The mixture was allowed to react for 3 min and then 300 µL of 1N Na_2CO_3 solution was added and mixed well. The solution was incubated in dark for 2 h at room temperature and the absorbance was measured at 725 nm using a spectrophotometer (UV-160A Shimadzu, Japan). The results were expressed as mg of gallic acid equivalent (GAE)/g dry weight, using a gallic acid standard curve.

Scavenging of DPPH radical

The free radical-scavenging activity of the obtained extracts was determined by the 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) assay as described (Yen and Chen 1995). DPPH (100 µM) was prepared in pure ethanol (99%). 1000 µL of DPPH solution was added to 100 µL of extracts (500 µg in ethanol) with 3900 µL of ethanol. The reactive mixture was shaken vigorously and allowed to stand for 10 min at room temperature in dark. The decrease in the absorbance of the resulting solution was monitored at 517 nm for 10 min. The results were corrected for dilution and expressed as µM of trolox equivalent (TE)/g of dry weight.

FRAP assay

Ferric reducing antioxidant potential of the extracts was determined by FRAP assay (Benzie and Strain 1996). A fresh working solution was prepared by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3.6H_2O$ solution and then warmed at 37°C before use. 100 µL of extracts (500 µg in ethanol), was allowed to react with 2900 µL of FRAP solution for 30 min in dark condition and the absorbance of the Ferrous tripyridyltriazine complex was measured at 593 nm. The results were corrected for dilution and expressed in mM of trolox equivalent (TE)/g of dry weight.

TPC and antioxidant activities of thymoquinone

The isolated thymoquinone and the synthetic antioxidants (BHA and α-tocopherol) were subjected to determination of TPC (Swain and Hillis 1959) and the antioxidant activities by DPPH method (Yen and Chen 1995) and FRAP assay (Benzie and Strain 1996) as previously mentioned in this Chapter.

Statistical analysis

The experiments were carried out in triplicate and the data calculated as means of ± SD along with a probability value of $P < 0.05$ was considered significant.

5.3. Results

Total phenolic content (TPC) of the extracts

The amount of total phenolics, varied widely from 25.41 to 77.40 mg GAE/g of extract on dry weight basis. The maximum total phenolic content of 77.40 ± 0.61 mg GAE/g was obtained at $SCCO_2$-1 (120 bar, 40°C) extract whereas methanol: water extract (70:30 v/v) showed a minimum total phenolic content of 25.41 ± 0.73 mg GAE/g, at 0.5 mg/mL concentration. According to the results, higher levels of pressure and temperature variations involved in the extraction procedures were found to minimize the total phenolic content of the extracts as presented in Fig 5.1. Among the obtained extracts, $SCCO_2$ extracts showed highest total phenolic content (TPC) which was followed by Soxtec™ extraction.

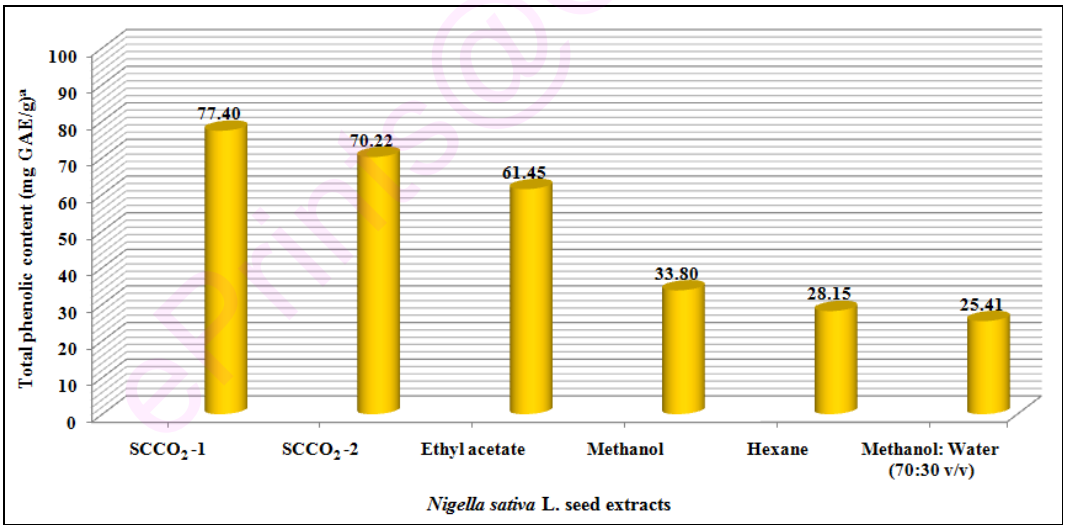


Fig. 5.1. Total phenolic content of *Nigella sativa* L. seed various extracts.
(SD values varied from 0.49-0.98).
[a]Total phenolics content were expressed as mg of gallic acid equivalent (GAE)/gram of extract.

DPPH free radical-scavenging assay of the extracts

The activity measured by the DPPH assay, ranged from 7.02 ± 0.36 to 84.23 ± 0.94 µM TE/g dry weight. DPPH assay measures the decrease in absorbance of the resulting solution which was monitored at 517 nm due to the formation of electron donating phenolic antioxidants. The various extracts of *Nigella sativa* seeds indicated extremely large variations in antioxidant activities which were evaluated by DPPH assay and the results are presented in Fig 5.2. In the present study, maximum DPPH activity of 84.23 ± 0.94 µM TE/g was obtained in $SCCO_2$-1 (120 bar, 40°C) extract while minimum activity of 7.02 ± 0.35 µM TE/g was obtained for methanol: water (70:30 v/v) extract, at 0.5 mg/mL concentration. Higher DPPH activity was found in $SCCO_2$ extracts than the conventional Soxtec™ extracts.

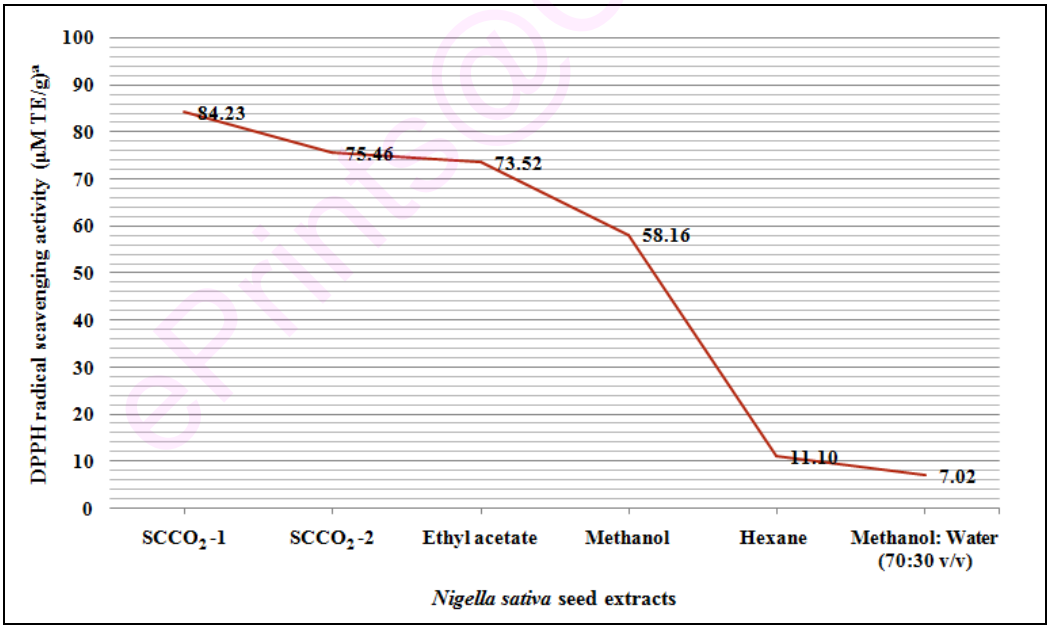


Fig. 5.2. DPPH free radical scavenging activity of *Nigella sativa* L. seed extracts. (SD values varied from 0.36-0.94).

[a]DPPH activity was expressed as µM trolox equivalent (TE)/gram of extract.

FRAP assay of the extracts

FRAP assay measures the change in the absorbance at 593 nm due to the formation of a blue colored ferrous tripyridyltriazine complex by the action of electron donating antioxidants. FRAP values ranged from 0.22 to 0.73 mM TE/g dry weight. In the present study, the $SCCO_2$-1 extract obtained at 120 bar pressure and 40°C temperature showed the maximum FRAP activity of 0.73 ± 0.03 mM TE/g whereas minimum activity of 0.22 ± 0.01 mM TE/g was found in methanol: water (70:30 v/v) extract, at 0.5 mg/mL concentration (Fig 5.3). $SCCO_2$ extracts were found to be having higher FRAP activity than the conventional Soxtec™ extracts.

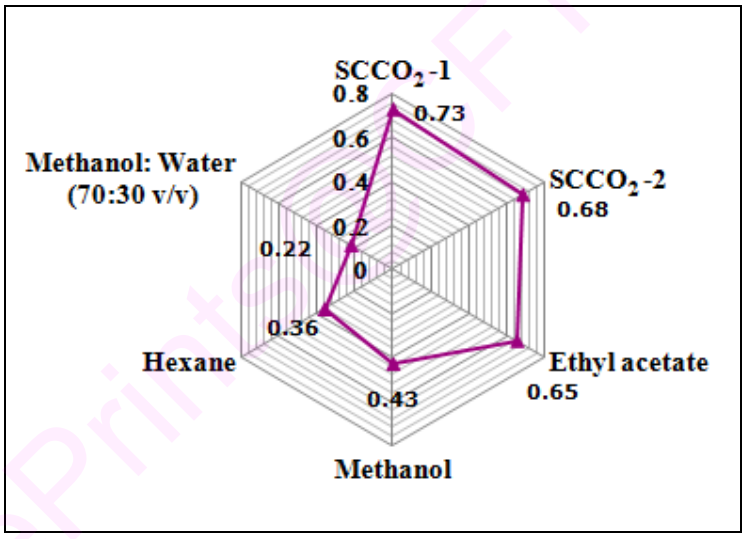


Fig. 5.3. FRAP activity of *Nigella sativa* L. seed extracts. FRAP activity was expressed as mM trolox equivalent (TE)/gram of extract. (SD values varied from 0.02-0.04)

The $SCCO_2$-1 extract showed higher TQ content (12.3 ± 0.24 %) along with rich amount of TPC (77.40 ± 0.61 mg GAE/g) and good antioxidant activities (DPPH value of 84.23 ± 0.94 µM TE/g and a FRAP value of 0.73 ± 0.03 mM TE/g) indicating the optimal performance of the extract. $SCCO_2$ extracts were found to have the least extractive yield though with higher TQ content than the Soxtec™ extracts.

The extractive yield and TQ content of all the extracts are presented earlier in Table 4.3 of Chapter 4. The $SCCO_2$ extracts were found to be of maximum TPC and maximum antioxidant activities in DPPH and FRAP assays than the conventional Soxtec^{TM} extracts (Figs. 5.1, 5.2 and 5.3).

TPC and antioxidant activities of thymoquinone

The higher total phenolic content (of 93.54 ± 1.65 mg of GAE/g) was recorded in isolated thymoquinone (TQ) was followed by the synthetic antioxidants (BHA and α-tocopherol). There was a significant difference in the value of the total phenolic content of TQ than that of synthetic antioxidants, as seen from the Table 5.1.

At DPPH assay, TQ compared to synthetic antioxidants prepared by ethanol (500 µg in ethanol) showed higher scavenging capacities. It showed higher scavenging activity (of 96.13 ± 1.29 µM TE/g) than the synthetic antioxidants. The absorbance of the DPPH radical owing to the scavenging capability of TQ and synthetic antioxidants is presented in Table 5.1.

In FRAP assay, the reducing power of TQ was higher than the synthetic antioxidants BHA and α-tocopherol. TQ has shown a higher reducing power (of 0.95 ± 0.04 mM TE/g) than the synthetic antioxidants (Table 5.1).

Table 5.1. Total phenolic content and antioxidant activities of isolated thymoquinone and synthetic antioxidants.

Compounds	Total phenolic content (mg GAE/g)[a]	DPPH (μM TE/g)[b]	FRAP (mM TE/g)[b]
Thymoquinone	93.54 ± 1.65	96.13 ± 1.29	0.95 ± 0.04
BHA	85.32 ± 1.15	90.22 ± 1.41	0.89 ± 0.03
α-tocopherol	88.70 ± 1.93	92.15 ± 1.20	0.91 ± 0.04

[a]Total phenolic content was expressed as mg of gallic acid equivalent (GAE)/gram of extract.

[b]Antioxidant activity was expressed as μM/ mM trolox equivalent (TE)/gram of extract.

The synthetic antioxidants BHA and α-tocopherol exhibited lower total phenolic content (TPC) as well as lesser antioxidant activities than the isolated thymoquinone (TQ). The TPC and antioxidant activity of TQ was higher than the crude extracts as well as the BHA and α-tocopherol (Figs. 5.1, 5.2 and 5.3; Table 5.1).

The obtained results revealed that the isolated thymoquinone (TQ) showed comparable levels of total phenolic content (TPC) and antioxidant activity to synthetic antioxidants BHA and α-tocopherol. TQ exhibited potent antioxidant activity which appears as a potential natural antioxidant agent, which confirmed TQ could be the antioxidant principle for *Nigella sativa* L. seed.

5.4. Discussion

In the present study, $SCCO_2$-1 extract yielded higher TQ content of 12.3%. The maximum total phenolic content was obtained by $SCCO_2$-1 (120 bar, 40°C and solvent to material ratio of 45 kg/kg) extract whereas methanol: water extract (70:30 v/v) showed minimum total phenolic content. High level of pressure and temperature variations involved in $SCCO_2$ extraction procedure was found to minimize the total phenolic content (TPC) of the extracts. The results thus indicate that the phenolic compounds especially the TQ which were extracted during the extraction process are thermo labile, photo reactive and volatile. Among the obtained extracts, $SCCO_2$ extracts showed highest total phenolic content (TPC) followed by the conventional Soxtec™ extracts. Such variations are mainly attributable to polarities of the different compounds present in *Nigella sativa* seeds. As reported earlier, there are varietal differences in total phenolic content values of *Nigella sativa* seed oil (Nejdet *et al.* 2010). And a comparison of the present study to this earlier report that there is a significant difference in the value of the total phenolic content between the seeds. There are reports that certain *Nigella sativa* seed oils have lesser total phenolic content than the present study owing to the varietal difference (Rababah *et al.* 2011; Souri *et al.* 2008). In the present study, the total phenolic contents were found to be (61.45, 33.80, 28.15 and 25.41 mg GAE/g) in conventional Soxtec™ extracts of ethyl acetate, methanol, hexane and methanol: water (70:30 v/v), respectively. Similar evidences are there for confirmation (Mariod *et al.* 2009).

In this study, TQ isolated from $SCCO_2$-2 extract of black cumin seeds which exhibited higher antioxidant activities on DPPH radicals than the synthetic antioxidants BHA and α-tocopherol. Similar findings are also reported in acetone

extract of black cumin showed good to excellent scavenging activity on DPPH radicals in comparison with positive BHA and BHT controls (Singh *et al.* 2005).

In the present investigation, the $SCCO_2$-1 extract showed higher FRAP activity (of 0.73 ± 0.03 mM TE/g). $SCCO_2$ extracts were found to be higher FRAP activity which was followed by conventional Soxtec^{TM} extracts. This is similar to the previously determined data that cumin belonging to Apiaceae family had higher FRAP capacity (of 1.83 ± 0.010g Trolox/100 g) indicating its higher antioxidant potential (Hossaina *et al.* 2008).

It is reported that the reducing properties are generally associated with the presence of reductones possessing the hydrogen-donating ability (Shimada *et al.* 1992; Barros *et al.* 2007). They showed antioxidant action by breaking the free radical chain via this property. Based on the reducing power results, the extract of the black cumin seed oil sample contain higher amounts of reductone compounds (i.e., carvacrol, thymol, 4-tertbutylcatechol, etc.) than the others (Nejdet *et al.* 2010). In our study also, it is established that the $SCCO_2$ extract of *Nigella sativa* seeds contain higher content of thymoquinone (TQ) as reductone compound, which is reflected its higher antioxidant potential.

There are reports that the $SCCO_2$ extracts of *Nigella sativa* seeds showed good antioxidant activity but with a lesser TQ content (Machmudah *et al.* 2005). In contrast to this, in the present study the $SCCO_2$ extracts showed higher antioxidant activity along with higher TQ content. This is also well supported by earlier findings (Erkan *et al.* 2008).

In our present study revealed that higher TQ content was noticed along with higher TPC and higher antioxidant activity. TQ content of the seeds may vary with plant species, variety, part of the plant, conditions of growth (soil, water and

temperature) and with the age of the plant. It also varies with geographical regions, seasons and time of collection and different climatic conditions (Reineccius 1994; Omidbaigi 1997; Gora *et al*. 2002). Though the TQ content of the seeds may be affected by the above mentioned reasons, extraction procedures also contribute to the level of availability of TQ content. In this study, $SCCO_2$ extracts were found to contain higher % of TQ than the conventional Soxtec™ extracts and its higher percentage might be attributed to higher antioxidant activity of those extracts.

In the present study, the higher TPC was recorded in isolated thymoquinone (TQ) followed by synthetic antioxidants (BHA and α-tocopherol). There was a significant difference in the value of TPC of isolated TQ than the synthetic antioxidants. At DPPH and FRAP assays, isolated TQ showed higher scavenging activity and higher reducing power followed by synthetic antioxidants.

Hence, the bioactive thymoquinone (TQ) from *Nigella sativa* seed appears to be promising as a potential natural antioxidant agent for commercial exploration.

5.5. Conclusion

It can be concluded that $SCCO_2$ extracts of *Nigella sativa* seed had higher antioxidant activity than conventional Soxtec™ extracts according to two different antioxidant assays (DPPH and FRAP). In addition, the antioxidant activity was found to be varying with different kinds of extraction solvents. Although the extract yield obtained by $SCCO_2$ is low, it contained higher TPC leading to higher antioxidant activities than the conventional Soxtec™ extracts. The various extracts are qualitatively and quantitatively affected by the type of extraction technique and solvents used. On the other hand, $SCCO_2$ extracts were found to contain higher % of TQ content. Hence, TQ is proved to be a potent natural antioxidant for food and pharmacological applications, replacing the synthetic antioxidants. Thymoquinone (TQ) exhibited potent antioxidant activity which appears as a natural antioxidant agent which confirmed it could be the antioxidant principle for *Nigella sativa* L. seed.

In view of the positive antioxidant and antibacterial potentials of the *Nigella sativa* L. seed extracts, especially the thymoquinone needs to be stabilized for future use as food and pharmacological applications.

CHAPTER 6

Enzymatic synthesis of thymol glycosides in $SCCO_2$ media

CHAPTER 6

6.1. Introduction

The putative and pharmacologically active four major quinonic phenol compounds thymoquinone (TQ), dithymoquinone (DTQ), thymohydroquinone (THQ) and thymol (THY) all possessing a thymol skeleton are present in *Nigella sativa* L. seeds as major phytochemicals. The presence of quinonic phenol phytochemicals in the *Nigella sativa* L. plant accounts for its chemical defence mechanism against phytopathogens. These thymol based quinonic phenol compounds play an important role in food, cosmetic and pharmacological applications and their relevant industries. Such an usage of thymol has certain limitation owing to its poor aqueous solubility, photosensitivity, thermal sensitivity and sublimation. To overcome these disadvantages, an attempt has been made for a novel synthesis of thymol glycosides by subjecting thymol to enzymatic glycosylation using diverse carbohydrates under $SCCO_2$ media. In this study, the enzyme fungal amyloglucosidase is used for getting thymol glycosides with an improved bioavailability and pharmacological activity for future applications.

Biotransformation

Biotransformation is considered to be an important tool for converting cheap and diverse organic compounds into more useful ones. The reactions involved in the biotransformation of organic compounds include oxidation, reduction, hydroxylation, esterification, methylation, isomerization, hydrolysis and glycosylation (Suga and Hirata 1990). Glycosylation is well known for improving the characteristics of food materials like enhancing solubility and reducing bitter after taste.

Enzymes are biocatalysts used increasingly in industries in recent times, particularly in cases, where chemical routes are difficult to be implemented (Johannes and Zhao 2006; Schoemaker *et al*. 2003). Oxido-reductases, hydralases (lipases,

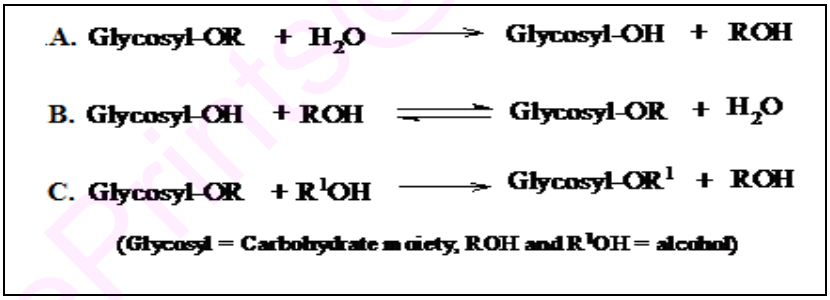
esterases, glycosidases, transglycosidases, peptidases, acylases, amidases, epoxide hydralases, nitrilases and hydantoinases), lyases and isomerases have been used in organic synthesis to a great extent (Nakamura and Matsuda 2002; Faber 2004). The conversion of substrate to product occurs at the active sites of an enzyme molecule. Presently, chiral compounds are the most important building blocks in chemical and pharmaceutical industries used for the production of flavours, agrochemicals and drugs (Daubmann *et al.* 2006). Interest for creating stereogenic centers by applying biocatalytic methods are on the rise these days (Davis and Boyer 2001; Honda *et al.* 2006; Fellunga *et al*. 2007; Szymanski *et al.* 2007; Arrigo *et al*. 2007; Lukowska and Plenkiewicz 2007; Zheng *et al*. 2007; Omori *et al.* 2007). Thus enzymes are powerful tools in the synthesis and modification of carbohydrate molecules used either alone or as whole cells (Goldberg *et al*. 2007a, 2007b).

Carbohydrates in the form of oligo and polysaccharides are universally found in nature and possess highly diverse biological functions. These compounds are obtained from simple cardohydrates by glycosyltransferases and are degraded by glycoside hydrolases and polysaccharide lyases. These types of enzymes known as carbohydrate active enzymes are key enzymes for clean processing of abundant and useful renewable resources. They are essential for biochemical studies in glycobiology as potential drugs due to their biocompatibility, structure forming capacity and environmentally benign properties (Allison and Grande Allen 2006; Volpi 2006; Yip *et al.* 2006; Prabakaran and Mano 2006).

Glycosylation

Glycosylation is the process or result of addition of carbohydrate molecules to proteins, aglycons and lipids. It is an enzyme directed site specific process as opposed to the non-enzymatic chemical glycation. Because of multiple hydroxyl groups of

similar reactivity, controlled glycosylation remains a challenge to organic chemists. Well elaborated and still widely employed classical chemical approaches inevitably require quite a number of protection, activation, coupling and deprotection steps (Akita *et al*. 1999; Konstantinovic *et al.* 2001). In contrast, glycosidases and transglycosidases offer one step synthesis under mild conditions in a regio and stereoselective manner (Vic and Thomas 1992; Vijayakumar and Divakar 2007; Vijayakumar *et al*. 2007). Enzyme-catalyzed glycosylation involves a glycosidase or a glycosyl transferase catalyzed glycoside bond formation. A carbohydrate nucleotide donor and acceptor are incubated with the appropriate glycosidase or glycosyl transferase that catalyzes the efficient and selective transfer of the glycosyl residue to the acceptor. Enzyme catalysed synthesis of glycosides can be acheived (Kadi and Crouzet 2008) through reverse hydrolysis (thermodynamically controlled) or transglycosylation (Scheme 6.1).



Scheme 6.1 (A, B, C). Reactions catalyze by glycosidase

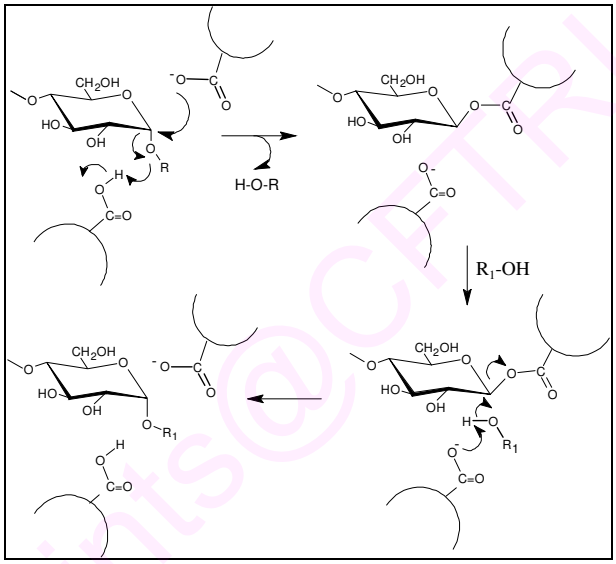
Glycosylation mechanism

During glycosidase catalysis, there are two possible stereochemical outcomes for the hydrolysis of a glycosidic bond - inversion and retension of anomeric configuration. Both the mechanisms involve oxocarbenium ion through transistion states and a pair of carboxylic acids at the active site. In inverting glycosidase, the reaction occurs via single displacement mechanism wherein one carboxylic group acts as a general base and the other as a general acid. In the retaining enzyme, the reaction

proceeds via double displacement mechanism (McCarter and Withers 1994; Wang *et al*. 1994). Site-directed mutagenesis continues to play a significant role in understanding mechanisms and an important role in the generation of enzymes with new specificities and possibly even new mechanisms (Faijes and Planas 2007).

Nucleophilic double displacement mechanism

The double displacement mechanism was found to be applicable to the enzymes, which retain the anomeric configuration of the substrate (Scheme 6.2).

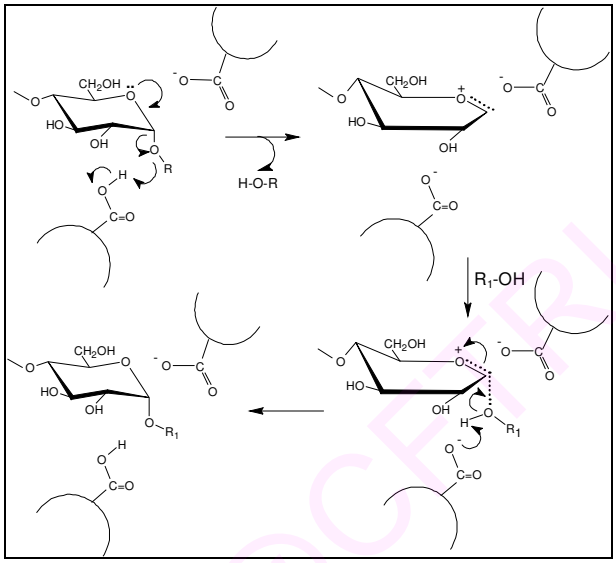


Scheme 6.2. Nucleophilic double displacement mechanism

The two catalytic ionisable groups, a carboxyl –COOH and a carboxylate, -COO^-, cleave the glucosidic linkage cooperatively by direct electrophilic and nucleophilic attacks against the glycosyl oxygen and anomeric carbon atoms respectively, resulting in a covalent glucosyl-enzyme complex through a single displacement. Subsequently glucosyl-acetal bond is attacked with the hydroxyl group of the water (alcohol hydroxyl group in glycosylation) by retaining the anomeric configuration of the product by the double displacement. The double displacement mechanism is adequate for explaining the reaction, where the anomeric configuration of the substrates is retained (Chiba 1997).

Oxocarbenium ion intermediate mechanism

In the oxo-carbenium intermediate mechanism, the two catalytic groups of the carboxyl and carboxylate ion participate cooperatively in the departure of the leaving group by a proton transfer to the anomeric oxygen atom (Scheme 6.3).



Scheme 6.3. Oxocarbenium ion intermediate mechanism

An enzyme bound oxonium ion intermediate has been detected by NMR (Withers and Street 1988). The second carboxylate, which is deprotonated in the resting state, stabilizes the oxonium ion intermediate. In the next step, a nucleophile adds to the same face of the glycosyl-enzyme intermediate from which the leaving group was expelled, resulting in the net retention of the anomeric configuration at the anomeric center. The addition of the nucleophile is assisted by the first carboxylate which in this step reverts to carboxylic acid. The oxo-carbenium intermediate mechanism has been applied to interpret the catalytic mechanism of many carbohydrate degrading enzymes. This mechanism is applicable to both 'retaining' and 'inverting enzymes'. Mutagenesis and X-ray structural studies have confirmed

that the mechanism of retaining glycosidases is similar (Sinnot 1990; Jacobson *et al.* 1994; 1995).

Advantages of enzymatic glycosylation over chemical methods

There are many advantages of using glycosidases (Takayama *et al*. 1997). High regio and stereoselectivity; Mild reaction conditions and biocompatibility; Replaces wasteful and expensive chemical procedure with more efficient semi-natural processes; Improved product-yield and better product quality; The use of non-polar solvents imparts stability to glycosidases and renders insolubility of the enzyme, solubility of alcohols and products in organic solvents and helps in arriving at easy product workout procedures; No protection, activation and deprotection steps are required and above all, it is environmental friendly.

Glycosidases

Glycosyl transferases, transglycosidases and phosphorylases are the enzymes responsible for glycosyl bond formation (Faijes and Planas 2007). Among the enzymes, glycosidases and transglycosidases play an important role in organic synthesis of glycosides. Starch degrading enzymes have been broadly classified into two groups - endo acting enzymes or endohydralases and exo-acting enzymes or exohydralases (Berfoldo and Anthranikian 2001). α-Amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) is an endo-acting enzyme, which is widely distributed in plant mammalia tissues and microorganisms (Maheswar Rao and Satyanarayana 2007). They catalyse the hydrolysis of α-1→4 glucosidic linkages of polysaccharides such as starch, glycogen or their degradation products (Tripathi *et al*. 2007). Exo acting starch hydrolases include β-amylase, glucoamylase, α-glucosidase and isoamylase. These enzymes attack the substrate from the nonreducing end, producing oligosaccharides. β-Amylase (1,4-α-D-glucan maltohydrolase, EC 3.2.1.2) is an exo-

acting enzyme which catalyzes the hydrolysis of 1,4-α-glycosidic linkages in starch, glycogen and related polysaccharides and oligosaccharides to remove successive β-maltose units from the non-reducing end of the chains. α-Glucosidase (EC 3.2.1.20) attacks α-1,4 linkages of oligosaccharides and liberates glucose with α-anomeric configuration.

Glucoamylase

Glucoamylase [α-(1,4)-D-glucan glucohydolase, EC 3.2.1.3] is a fungal enzyme also known as amyloglucosidase, maltase, saccharogenic amylase and γ-amylase belonging to an important group of starch degrading enzymes (Riaz *et al*. 2007). They catalyze the hydrolysis of α-1,4 and α-1,6 glycosidic linkages from the non-reducing end of starch and related oligosaccharides, the α-1,6 activity rate is only 0.2% of that of α-1,4 (Meages *et al*. 1989; Jafari Aghdam *et al*. 2005) with the inversion of anomeric configuration to produce β-glucose (Norouzian *et al*. 2006; Thorsen *et al*. 2006). Reverse reactions involving synthesis of saccharides and glycosides from D-glucose occur with a very high glucoamylase concentration, prolonged incubation periods and high concentration of substrates. Moreover, these are extensively used in the production of different antibiotics and amino acids in brewing, textile, food, paper and pharmaceutical industries (Mamo and Gessesse 1999; Marlida *et al*. 2000; Sanjay and Sugunan 2005). Thermostability and near neutral pH activity are some of the properties which can largely benefit the starch industry and therefore have been the areas of great interest in glucoamylase research (Ford 1999; Vieille and Zeikus 2001). *Rhizopus oryzae* was reported as being capable of simultaneously saccharifying and fermenting cornstarch and other cereals to L-lactic acid (Yu and Hang 1991; Suntornsuk and Hang 1994).

Structural features of glucoamylase

Glucoamylase family consists of a starch binding domains attached to each other by means of an *O*-glycosylation linker (Saver *et al*. 2000; Coutinho and Reilly 1997; Horvathova *et al*. 2001). Structures of a family of 21 carbohydrate binding modules from starch binding domain of *Rhizopus oryzae* glucoamylase determined by NMR spectroscopy explains ligand binding sites and structural features of this glucoamylases (Liu *et al*. 2007). The enzyme contains a very specific carbohydrate region consisting of 30 chains in the form of di- or trisaccharides (Wang *et al*. 1994). Also, it contains three domains (Svensson *et al*. 1983): N-terminal catalytic domain (residues 1 to 470, 55 kDa), a short bulky linker (residues 471 to 508, 13 kDa) which is heavily *O*-glycosylated at the abundant serine and threonine residues and C-terminal granular starch binding domain (residues 509 to 616, 12 kDa). The bulky linker joins the two main domains giving the enzyme an overall dumb-bell shape (Kvamer *et al*. 1993). Starch binding domain glucoamylase I from *A. niger* shows a well defined β-sheet structure consisting of one parallel and six antiparallel pairs of β-strands which forms an open sided β-barrel (Sorimachi *et al*. 1996). Three forms of glucoamylase were isolated from *Rhizopus* sp., Glu1, Glu2 and Glu3. Various forms of glucoamylases are thought to be the result of several mechanisms: mRNA modifications, limited proteolysis, variation in carbohydrate content or the presence of several structural genes (Pretorius *et al.* 1991; Takahashi *et al.* 1985; Vihinen and Mantsala 1989).

The structure of different glucoamylases showed a common subsite arrangement, seven in total and the catalytic site was located between subsites 1 and 2 (Ohinishi 1990; Fagerstrom 1991; Ermer *et al.* 1993). Subsite 2 has the highest affinity for oligomeric substrates and glucose, followed by decreasing affinity towards

subsites 3 to 7 (Fagerstrom 1991). Complexes of glucoamylase from *A. awamori* with acarbose and D-gluco-dihydroacarbose indicate hydrogen bonds between carbohydrate OH groups and Arg54, Asp55, Leu177, Try178, Glu180 and Arg305 involved in binding at subsites 1, 2 and an array of outer subsites leading into these inner ones (Aleshin *et al.* 1994; Stoffer *et al.* 1995). The geometry of the general acid and base catalysis of Glu179 (Sierks *et al.* 1990; Aleshin *et al.* 1992) and Glu400, is excellent for the glucoside bond cleavage and assistance in the nucleophilic attack of water at the anomeric center of the carbohydrate (Harris *et al.* 1993; Frandsen *et al.* 1994). The active site of *A. niger* glucoamylase is very much identical to that of the *Rhizopus oryzae* (Stoffer *et al.* 1995). In the active site of *R. oryzae,* the aminoacid residues Arg191, Asp192, Leu312, Trp313, glu314, Glu315 and Arg443 are responsible for substrate binding through hydrogen bonds where as Glu314 and Glu544 are for glucosidic bond cleavage (Ashikari *et al.* 1986; Sierks *et al.* 1990; Aleshin *et al.* 1992).

Glycosides

Glycosides are widely distributed in nature and can be found in nearly every living organism (Roode *et al.* 2003). Glycosides are asymmetric mixed acetals formed by reactions of the anomeric carbon atom of intermolecular hemiacetal or pyranose or furanose form of the aldohexose or aldopentose or ketohexose with a hydroxyl group furnished by an alcohol. In an aqueous media, when there is large excess of water, hydrolysis of glycoside or oligosaccharide or polysaccharide is the dominant reaction (Scheme 6.1A). In case of reverse hydrolysis and transglycosylation reactions, synthesis of glycosides using glycosidases or transglycosidases depends on the nature of the glycosyl donor and the native of the medium. The reverse hydrolytic approach is an equilibrium controlled synthesis where the equilibrium is shifted towards

synthesis (Panintrarux *et al.* 1995; Vic *et al.* 1997; Rantwijk *et al.* 1999; Biely 2003) involving a carbohydrate and an alcohol (Scheme 6.1B). This can be achieved by reducing the water activity and by increasing the substrate concentrations so that the substrate itself acts as a solvent media (Vic and Crout 1995). This method is widely employed for the enzymatic synthesis of alkyl glycosides and phenolic glycosides in an organic solvent or co-solvent (Vic and Crout 1995; Vic *et al.* 1997; Ducret *et al.* 2002; Vijayakumar and Divakar 2005). The transglycosylation method is a kinetically controlled synthesis where the enzyme catalyzes the transfer of a glycosyl residue from a glycosyl donor to the glycosyl acceptor (Scheme 6.1C). The reaction yield depends on the rate of product synthesis relative to the rate of hydrolysis. An efficient acceptor used in a high concentration should favor the synthesis (Ismail *et al.* 1999; Rantwijk *et al.* 1999; Eneyskaya *et al*. 2003; Jiang *et al*. 2004; Tsuruhami *et al*. 2005).

Properties of glycosides (Stanek *et al.* 1963)

1. Glycosides are readily soluble in water and crystallize easily.
2. Their aqueous solutions, if not attacked by microorganisms, are stable, do not exhibit mutarotation and are non-reducing.
3. The circumstance that water insoluble substances are rendered soluble by their linkage to carbohydrate is of great importance for the detoxification of many phenolic compounds which has application in industries and pharmaceuticals.
4. In contrast to free carbohydrate, the glycosides are not sweet (apart from a few exceptions such as methyl α-D-glucopyranoside). They taste more or less bitter.
5. Glycosides are split by acids even at low concentrations. The rate of hydrolysis depends on the anomeric configuration, nature of aglycon, type of carbohydrate and cyclic arrangement.

6. Unsubstituted glycosides are less sensitive to oxidative agents than free carbohydrate.
7. The glycosides are also cleaved by the action of enzymes, which are generally termed glycosidases. The reaction is a reversible one and under suitable conditions they may also be used for the synthesis of glycosides from free carbohydrate components and aglycons.

Applications of glycosides

Glycosides exhibit a wide variety of applications. Carbohydrate connected to long alkyl chains as aglycons yield glycosides with good surfactant and emulsifying properties and therefore used in detergents (Katusumi *et al*. 2004; Larsson *et al*. 2005) and cosmetics (Luther *et al.* 1999). Glycosides with terpenes are claimed to possess antifungal and antimicrobial activities (Zhou 2000). Glycosides of flavour and fragrances are used in perfumery (Watanabae *et al.* 1994; Odoux *et al*. 2003). In plants, glycosides are believed to play an important role in accumulation, storage and transport of hydrophobic substances. Cyanogenic glycosides play a role in plant defense mechanism. Table 6.1 lists some of the important phenolic glycosides and its applications.

Table 6.1. Phenolic glycosides synthesized through enzymatic glycosylation with respective enzyme source and their applications

Name of the compound	Source of enzyme	Applications	References
Phenolic glycosides Eugenol-5-*O*-β-(6′-galloylglucopyranoside)	*Melaleuca ericifolia*	Antibacterial	Hussein *et al*. 2007
Eugenol-α-glucoside	α-Glucosyltransfer enzyme of *Xanthomonas campestris* WU-9701	As a prodrug of a hair restorer, as a derivative of spices	Sato *et al.* 2003
Eugenol-β-glucoside	Biotransformation by cultured cells of *Eucalyptus perriniana*	As a prodrug of a hair restorer	Orihara *et al.* 1992
Eugenyl-α-D-glucoside, eugenyl-α-D-mannoside, eugenyl-maltoside, eugenyl-sucrose, eugenyl-mannitol	Amyloglucosidase, almond β-glucosidase	Antioxidant	Vijayakumar and Divakar 2007
Vanillin-β-D-monoglucopyranoside	Cell suspension culture of *Coffea arabica*	As a food additive flavor	Kometani *et al.* 1993a, Odoux *et al*. 2003
Capsaicin-β-D-glucopyranoside	Cells suspension culture of *Coffea arabica* Cultured cells of *Phytolacca americana*	Food ingredient and pharmacological applications	Kometani *et al.* 1993b Hamada *et al.* 2003
Capsaicin-4-*O*-(6-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside, capsaicin-4-*O*-(6-*O*-α-L-arabinopyranosyl)-β-D-glucopyranoside, 8-nordihydrocapsaicin-4-*O*-(6-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside, 8-nordihydrocapsaicin-4-*O*-(6-*O*-α-L-arabinopyranosyl)-β-D-glucopyranoside,	Cultured cells of *Catharanthus roseus*	Food, spices and medicines	Shimoda *et al*. 2007
α-Salicin, α-isosalcin, β-salicin	*Bacillus macerans* cyclodextrin glucanyl transferase and *Leuconostoc mesenteroides* B-742CB dextransucrase	Anti-inflammatory, analgesic antipyretic prodrug	Yoon *et al.* 2004
Curcumin glucosides, Curcumin-4′-4″-*O*-β-D-digentiobioside	Cell suspension cultures of *Catharanthus roseus*	Food colorant, antioxidant, anticancer	Kaminaga *et al.* 2003

Curcuminyl-bis-α-D-glucoside, curcuminyl-bis-α-D-mannoside, curcuminyl-bis-maltoside, curcuminyl-bis-sucrose, curcuminyl-bis-mannitol	Amyloglucosidase	Food colorant, antioxidant	Vijayakumar and Divakar 2007
Echinacoside, aceteoside, 2′-acetyl acteoside, cistanoside A, cistanoside B	Plant cell culture of *Cistanche deserticola*	Antinociception, anti-inflammatory, sedation	Ouyang *et al*. 2005
Elymoclavine-*O*-β-D-fructofuranoside	Saprophytic culture of *Claviceps* sp.	In the treatment orthostatic circulary disturbances, hyperprolactinemia, antibacterial and cytostatic effects and hypolipemic activity	Ken and Cvak 1999
2-Hydroxybenzyl-β-D-galacto pyranoside, 3-hydroxy benzyl-β-D-galactopyranoside, 4-hydroxybenzyl-β-D-galactopyranoside, 3-aminobenzyl-β-D-galacto pyranoside, 3-(hydroxymethyl)-aminobenzyl-β-D-galactopyranoside, [3-(2-methoxy)-2-hydroxy propanol]-β-D-galactopyranoside, [3-(4-chloro phenoxy)-2-hydroxypropanol]-β-D-galacto pyranoside, 3-methoxybenzyl-β-D-galacto pyranoside, 1-phenylethyl-β-D-galactopyranoside.	β-galactosidase from *Kluyveromyces lactis*	Therapeutic agent	Bridiau *et al*. 2006

Glycosylation in supercritical carbon dioxide

Several reviews describe the variety of organic reactions including chemical (hydrogenation, hydroformylation, photorection, halogenation, Diels Alder cycloaddition, oxidation, coupling reaction, Pauson-Khand reaction, olefin methathese, Friedel-Craft alkylation, asymmetric reaction) and enzymatic reactions carried out in supercritical fluids (Mori and Okahata 2000, 2002; Oakes *et al.* 2001; Matsuda *et al.* 2002).

Current research has provided insight into the advantages and possibilities of using supercritical carbon dioxide ($SCCO_2$) in biochemical processes (Dijkstra *et al*. 2007). The main advantages of $SCCO_2$ include increased catalytic activities as a result of improved mass transfer, higher selectivities and strongly reduced organic waste streams (De Simone 2002; Beckmann 2003; Rezaci *et al*. 2007).

Carbon dioxide was chosen as the supercritical fluid (SCF) as CO_2 becomes a SCF above 31°C and 73.8 atm, which are easily accomplished conditions with gentle heating from ambient temperature and a commercial liquid chromatography pump. The solvent properties of $SCCO_2$ can be continuously varied by changing the pressure or temperature. Moreover CO_2 is nontoxic and the medium is easily removed by decompression to atmospheric pressure (Mori and Okahata 1998, 2000).

Enzyme stability can be improved by $SCCO_2$ pretreatment. The application of supercritical fluids in the control of enzyme reactions, with emphasis on the use of supercritical fluroform in the regulation of β-D-galactosidase mediated transglycosylation and oxidation has been discussed (Mori and Okahata 2003). $SCCO_2$ pretreated α-amylase retained 41% activity based on the original activity, whereas, the non-treated α-amylase completely lost its activity in an hour in water (Liu and Cheng 2000). n-Octyl-β-D-

xylotrioside and xylobioside synthesis were significantly increased in supercritical CO_2 and fluroform (CHF_3) fluids mediated one-step reaction of xylan and n-octanol using the acetone powder (acetone-dried cells) of *A. pullulans* as the enzyme source of xylanase (Nakamura *et al.* 2000; Matsumura *et al.* 1999). A lipid-coated β-galactosidase catalyzed transgalactosylation reactions carried out in $SCCO_2$ resulted in good conversion yields up to 72%, whereas, native β-galactosidase hardly catalyzed the transgalactosylation in $SCCO_2$ due to its insolubility and instability in $SCCO_2$ (Mori and Okahata 1998).

Thymol

A pungent-smelling colourless (white) crystalline aromatic compound, $C_{10}H_{14}O$, derived from *Nigella sativa* L. seed oil, thyme oil and other oils or made synthetically and used as an antiseptic, a fungicide, and a preservative. Thymol occurs in various essential oils, particularly in the oil of black cumin and thyme, and can be made by using iron (III) chloride to oxidize piperitone (itself extracted from eucalyptus oil). Its antiseptic properties are exploited in gargles and mouthwashes. Thymol is an aroma compound present in the essential oil of *Nigella sativa* L. seeds. It is produced by these plant species as a chemical defence mechanism against phytopathogens (Vazquez *et al.* 2001). Therefore, this compound has attracted much attention in food industry, as it has been used in foods such as cheese as natural preservative to prevent fungal growth. (Juven *et al*. 1994; Vazquez *et al.* 2001; Venturini *et al*. 2002). Thymol and its studied product thymoquinone has also been used in medicine because of its pharmacological importance as antiseptic, antispasmodic, tonic and carminative (Didry *et al*. 1994). Irrespective of such biological and pharmacological activities, its use is restricted,

because of its poor aqueous solubility (1g in 1L) (The Merck index 2006), sublimation, light and heat sensitivity (Ghosheh *et al.* 1999).

From the physiological point of view, the glycosides of thymol can be of pharmacological interest as well as in use in cosmetics and in food as additives. Several attempts have been made to produce thymol glycosides by different methods like chemical glycosylation to yield β-glucosides (Mastelic *et al*. 2004). Shimoda *et al*. (2006) reported that glucosyltransferases in the cultured cells of *Eucalyptus perriniana* have the highest specificity for thymol as substrate and are able to convert thymol into higher water soluble β-glucoside and β-gentiobioside which are accumulated in the cells.

Glycosylation occurs readily in plant cells to produce secondary metabolites like saponins and anthocyanins in the form of glycosides in higher plants (Furuya *et al.* 1989). Glycosylation, besides being an important versatile method for the structural modification of compounds with valuable biological activities, also allows the conversion of water-insoluble and unstable organic compounds into the corresponding water-soluble and stable ones, thereby enriching their pharmacological applications (Suzuki *et al.* 1996; Vijayakumar and Divakar 2005; Sivakumar and Divakar 2006).

A one-step enzymatic glycosylation is useful for the synthesis of thymol glycosides rather than chemical glycosylation, which requires many tedious protection-deprotection steps. Enzymatic glycosylation of thymol with different carbohydrates using supercritical fluid media, has not been reported so far.

The aim of the present study deals with novel synthesis of thymol glycosides with diverse carbohydrate molecules using amyloglucosidase from *Rhizopus* mold under $SCCO_2$ conditions (Fig 6.1).

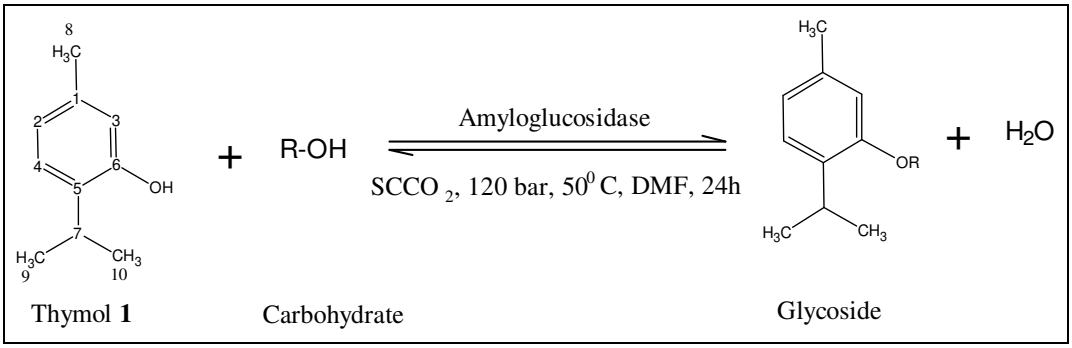


Fig. 6.1. Synthesis of thymol glycosides.

This investigation attempts to explore the potentialities of amyloglucosidase from *Rhizopus mold* in effecting glycosylation of a wide variety of glycosyl acceptors (of D-glucose, D-galactose, D-mannose, D-fructose, D-arabinose, D-ribose, maltose, sucrose, lactose, D-mannitol and D-sorbitol) under $SCCO_2$ conditions.

Amyloglucosidase (from *Rhizopus* sp.) catalyzed syntheses of water soluble thymol glycosides were carried out in dimethyl formamide (DMF) under $SCCO_2$ conditions where larger concentration of substrates can be employed with lesser concentration of enzyme to get better conversion. Glycosides prepared were analysed by HPLC, separated through size exclusion chromatography and characterized spectroscopically to determine their nature and proportions. Thymol was glycosylated with a specific aim for expected bioavailability and enhanced pharmacological activity.

6.2. Materials and methods

Enzyme and chemicals

Amyloglucosidase (EC 3.2.1.3) from *Rhizopus* mold (Cat. No. A-7255; Lot. No. 122K1561 from Sigma Chemical Company, USA) and thymol (Aldrich Chemical Company, USA). D-galactose and D-fructose (HiMedia Pvt. Ltd, India), D-mannose, D-ribose, D-arabinose, glacial acetic acid and sodium azide (Loba Chemie Pvt. Ltd, India); DMF (Sisco Research Laboratories Pvt. Ltd, India); Bio-Gel P-2 Gel (Bio-Rad Laboratories, Inc. USA); Silica gel G for thin layer chromatography (Sd fine-Chem Limited, India); n-butanol (Qualigens fine chemicals, India); DMSO-d_6 (Merck Co, India) and food grade CO_2 cylinder (99.9% purity, Kiran Corporation, Mysore, India) were purchased and employed in this study. Amyloglucosidase activity was found to be 11.2 AU/μmol/mg/ min/enzyme (Sumner and Sisler 1944).

Synthesis of thymol glycosides under $SCCO_2$

Glycosylation of thymol **1** (6.0 mmol) involved reaction with 6.0 mmol of carbohydrates (D-galactose **2**, D-mannose **3**, D-fructose **4**, D-ribose **5** and D-arabinose **6**) in 10 ml DMF in presence of 40% (w/w carbohydrates corresponding to 680—810 μmol per min per mg of enzyme proportion in terms of hydrolytic activity units) of amyloglucosidase and 3.0 ml of phosphate buffer (0.01 M, pH 6.0) under $SCCO_2$ conditions of 120 bar pressure at 50°C for 24 h. A CO_2 reactor vessel of 120 ml capacity with a magnetic stirrer, thermostatically controlled to attain a constant temperature was employed (Figs 6.2 and 6.3).

Fig. 6.2. Reaction setup for glycosylation of thymol in supercritical CO_2

After the reaction, CO_2 was released and the reacted products were extracted with 15-20 ml of distilled water which was later evaporated. The enzyme was denatured at 100°C in a boiling water-bath for 5-10 min and the dried residue was subjected to chromatographic separation. Unreacted carbohydrate was separated from the product glycosides by size exclusion chromatography using Bio-gel P-2 column (100 cm × 1 cm)

packed along with 0.05 mg of sodium azide to prevent bacterial contamination and eluted with distilled water at 1 ml/h rate. Column fractions were continuously monitored by Silica gel-G coated TLC plates with n-butanol: glacial acetic acid: distilled water (75:15:10 v/v/v) as developing solvent. Individual glycosides could not be separated well, because of similar polarity of the glycosides formed. The dried residue was subjected to HPLC analysis on an aminopropyl column (3.9 mm × 300 mm length), using acetonitrile: water (80:20 v/v) as a mobile phase at 1 ml/min flow rate and monitored with a refractive index detector.

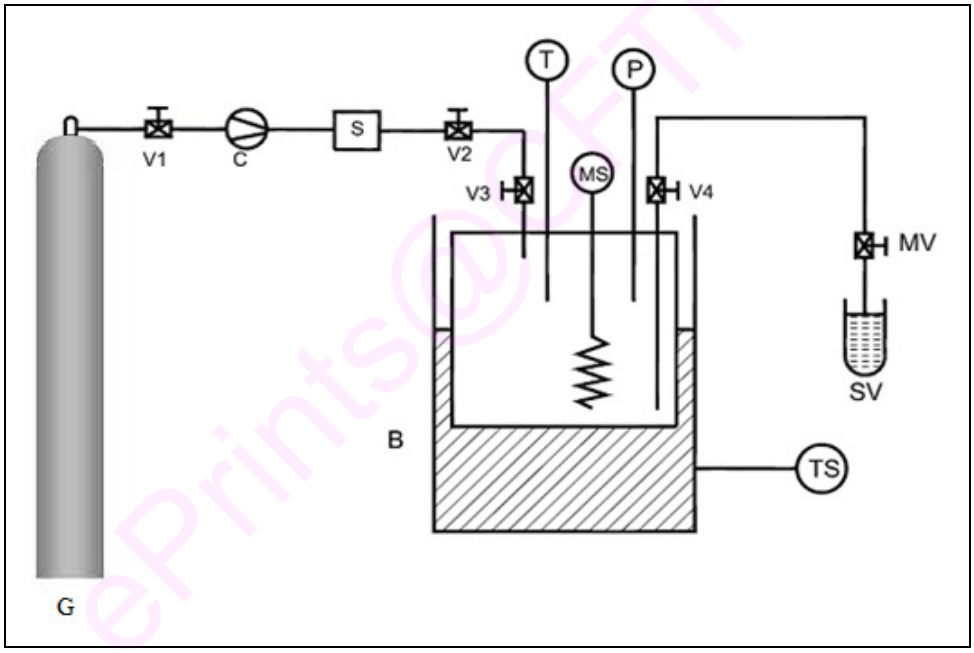


Fig. 6.3. Schematic diagram of reaction setup for thymol glycosylation in supercritical CO_2

G: Gas cylinder	T: Temperature indicator	MV: Micrometer valve
C: Compressor	P: Pressure indicator	SV: Sampling valve
S: Surge tank	MS: Magnetic stirrer	V1-V4: High pressure needle valve
B: Berghof autoclave	TS: Thermostat	

The Retention times for the substrates and products were: D-galactose-9.6 min, D-mannose-8.1 min, D-fructose-8.0 min, D-ribose-5.3 min and D-arabinose-7.3 min, thymol-6-*O*-D-galactopyranoside-9.1 min, thymol-6-*O*-D-mannopyranoside-8.7 min (Fig.6.4), thymol-6-*O*-D-fructofuranoside-7.8 min, thymol-6-*O*-D-ribofuranoside-5.7 min and thymol-6-*O*-D-arabinofuranoside-7.0 min.

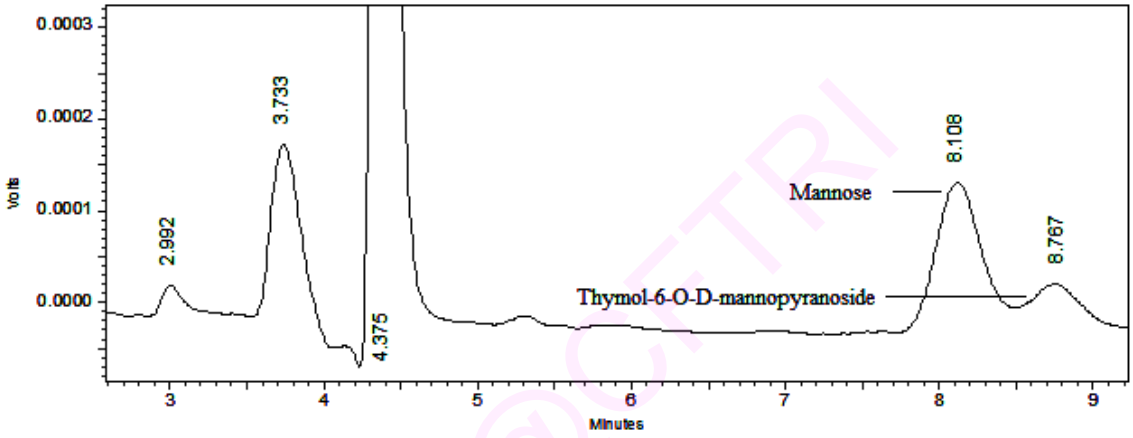


Fig. 6.4. HPLC chromatogram of thymol-6-*O*-D-mannopyranoside **8a-b**

The conversion yields were determined from HPLC peak areas of the glycosides and carbohydrate peaks and expressed as percentage glycosides formed with respect to the concentration of the carbohydrates **2–6** employed. The extent of formation of individual glycosides was determined from H-1/H-6 and C1/C6 peak areas from the ^{1}H and ^{13}C NMR 2D spectra (Charles *et al.* 2009). Error in yield measurements was ± 5–10%. All the yields were an average from two independent experiments as the yields were within ± 5% of error.

Spectral characterization

Isolated thymol glycosides were characterized spectroscopically by UV, IR, Mass and Two-Dimensional Heteronuclear Single Quantum Coherence Transfer (2D HSQCT) NMR spectra which provided good information about the nature and type of products. The Mass spectra were obtained using a Q-TOF Waters Ultima instrument (Waters Corporation, Manchester, UK) fitted with electron spray ionization (ESI) source. 2D HSQCT were recorded along with ^{1}H and ^{13}C NMR spectra on a Bruker Avance AQS-500 MHz (Bruker Biospin, Fallanden, Switzerland) NMR spectrometer (500.13 MHz for ^{1}H and 125 MHz for ^{13}C) at 35°C using 40 mg of the isolated glycosides in DMSO-*d6* solvent. In the NMR data, only resolvable signals are shown. Some of the assignments are interchangeable. The isolated glycosides, being surfactant molecules tend to aggregate in solution giving rise to broad signals, thus making it difficult to resolve the coupling constant values of some of the proton signals. Hence, in the proton part of 2D HSQCT, coupling constant values including those of few anomeric protons could not be resolved satisfactorily.

Thymol 1

Solid, mp 51.5°C; UV (alcohol, λ_{max}): 276 nm ($\pi \rightarrow \pi^*$, ε_{276}-2320 M^{-1}); IR (KBr) (stretching frequency): 3610 cm^{-1} (OH), 2970 cm^{-1} (aromatic CH), 1580 cm^{-1}(C=C); MS (*m/z*) 150.2 $[M]^+$; 2D HSQCT (DMSO-d_6) ^{1}H NMR δ_{ppm} (500.13): 7.47 (H-2, 8.0 Hz), 6.72 (H-3), 7.85 (H-4, 8.0 Hz), 2.01 (H-7, 5.4 Hz), 1.37 (H-9, 5.4 Hz), 1.95 (H-10, 5.4 Hz); ^{13}C NMR δ_{ppm} (125 MHz): 127.9 (C2), 133.5 (C3), 125.8 (C4), 127.9 (C5), 154.1 (C6), 30.8 (C7), 23.5 (C9), 26.2 (C10).

Thymol-6-*O*-D-galactopyranoside 7 a-b

Solid, Isolated yield 375 mg (20.0%); UV (λ_{max}): 283 nm ($\pi\rightarrow\pi^*$, ε_{283}– 510 M^{-1}); IR (KBr) (stretching frequency): 3390 cm^{-1} (OH), 1250 cm^{-1} (glycosidic aryl alkyl C-O-C asymmetrical), 1070 cm^{-1} (glycosidic aryl alkyl C-O-C symmetrical); MS (*m/z*) 311.93 $[M-1]^+$; 2D HSQCT (DMSO-d_6) **C1α-galactoside 7a**: ^{1}H NMR δ_{ppm} (500.13): **Gal**: 4.97 (H-1α), 3.88 (H-2α), 3.68 (H-3α), 3.63 (H-4α), 3.32 (H-6α); ^{13}C NMR δ_{ppm} (125 MHz): **Gal**: 95.5 (C1α), 71.1 (C2α), 77.6 (C3α), 69.0 (C4α), 73.3 (C5α), 61.4 (C6α). **C1β-galactoside 7b**: ^{1}H NMR δ_{ppm} (500.13): **Gal**: 4.91 (H-1β), 3.78 (H-2β), 3.82 (H-3β), 3.52 (H-4β), 3.93 (H-5β), 3.35 (H-6β); ^{13}C NMR δ_{ppm} (125 MHz): **Gal**: 101.9 (C1β), 70.8 (C2β), 76.4 (C3β), 69.1 (C4β), 74.8 (C5β), 62.8 (C6β).

Thymol-6-*O*-D-mannopyranoside 8 a-b

Solid, Isolated yield 263 mg (14.0%); UV (λ_{max}): 286 nm ($\pi\rightarrow\pi^*$, ε_{286}–490 M^{-1}); IR (KBr) (stretching frequency): 3340 cm^{-1} (OH), 1255 cm^{-1} (glycosidic aryl alkyl C-O-C asymmetrical), 1065 cm^{-1} (glycosidic aryl alkyl C-O-C symmetrical), 2940 cm^{-1} (CH); MS (*m/z*) 311.93 $[M-1]^+$; 2D HSQCT (DMSO-d_6) **C1α-Mannoside 8a**: ^{1}H NMR δ_{ppm} (500.13): **Man**: 3.0 (H-3α), 3.40 (H-6α); **Thymol**: 2.90 (H-7); ^{13}C NMR δ_{ppm} (125 MHz): **Man**: 78.1 (C3α), 63.5 (C6α); **Thymol**: 135.4 (C2), 128.0 (C3), 129.8 (C4), 154.2 (C6), 36.0 (C7), 24.6 (C8), 31.0 (C9), 31.0 (C10). **C1β-Mannoside 8b**: ^{1}H NMR δ_{ppm} (500.13): **Man**: 3.58 (H-3β), 3.35 (H-4β), 3.01 (H-5β), 3.40 (H-6β); **Thymol**: 3.60 (H-3), 3.52 (H-4), 2.90 (H-7); ^{13}C NMR δ_{ppm} (125 MHz): **Man**: 101.7 (C1β), 74.9 (C2β), 79.0 (C3β), 67.2 (C4β), 77.2 (C5β), 63.5 (C6β).

Thymol-6-*O*-D-fructofuranoside 9 a-b

Solid, Isolated yield 506 mg (27.0%); UV (λ_{max}): 290 nm ($\pi\rightarrow\pi^*$, ε_{290}–340 M^{-1}); IR (KBr) (stretching frequency): 3430 cm^{-1} (OH), 1250 cm^{-1} (glycosidic aryl alkyl C-O-C asymmetrical), 1060 cm^{-1} (glycosidic aryl alkyl C-O-C symmetrical), 2940 cm^{-1} (CH); MS (*m/z*) 312.80 $[M]^+$; 2D HSQCT (DMSO-d_6) **2-*O*-D-fructoside 9a**: ^{1}H NMR δ_{ppm} (500.13): **Fru**: 3.12 (H-1), 4.30 (H-3), 3.65 (H-4), 3.78 (H-5), 3.41 (H-6); ^{13}C NMR δ_{ppm} (125 MHz): **Fru**: 64.6 (C1), 104.2 (C2), 76.1 (C3), 69.3 (C4), 83.0 (C5), 63.9 (C6); **Thymol**: 154.2 (C6), 35.9 (C7), 30.9 (C9), 30.9 (C10). **1-*O*-D-fructoside 9b**: ^{1}H NMR δ_{ppm} (500.13): **Fru**: 3.22 (H-1), 4.28 (H-3), 3.78 (H-4), 3.72 (H-5), 3.32 (H-6); **Thymol**: 2.88 (H-7); ^{13}C NMR δ_{ppm} (125 MHz): **Fru**: 61.3 (C1), 98.1 (C2), 76.0 (C3), 64.5 (C4), 81.2 (C5), 59.0 (C6); **Thymol**: 154.2 (C6), 35.9 (C7), 30.9 (C9), 30.9 (C10).

Thymol-6-*O*-D-ribofuranoside 10 a-b

Solid, Isolated yield 190 mg (11.2%); UV (λ_{max}): 278 nm ($\pi\rightarrow\pi^*$, ε_{278}–380 M^{-1}); IR (KBr) (stretching frequency): 3480 cm^{-1} (OH), 1050 cm^{-1} (glycosidic aryl alkyl C-O-C symmetrical), 2915 cm^{-1} (CH); MS (*m/z*) 282.01 $[M]^+$; 2D HSQCT (DMSO-d_6) **C1α-riboside 10a**: ^{1}H NMR δ_{ppm} (500.13): **Rib**: 4.70 (H-1α), 3.40 (H-2α); 3.50 (H-3α), 3.90 (H-4α), 3.65 (H-5α); **Thymol**: 7.68 (H-3), 7.65 (H-4), 2.5 (H-7), 1.25 (H-9), 1.28 (H-10); ^{13}C NMR δ_{ppm} (125 MHz): **Rib**: 93.8 (C1α), 69.5 (C2α), 67.3 (C3α), 71.0 (C4α), 60.7 (C5α); **Thymol**: 135.5 (C2), 128.8 (C3), 131.7 (C4) 29.9 (C7), 23.4 (C8), 29.1 (C9), 28.6 (C10). **C1β-riboside 10b**: ^{1}H NMR δ_{ppm} (500.13): **Rib**: 5.05 (H-1β), 3.20 (H-2β), 3.80 (H-4β), 3.30 (H-5β); ^{13}C NMR δ_{ppm} (125 MHz): **Rib**: 96.3 (C1β), 70.4 (C2β), 83.3 (C4β), 61.8 (C5β).

Thymol-6-*O*-D-arabinofuranoside 11a-b

Solid, Isolated yield 362 mg (21.4%); UV (λ_{max}): 307 nm ($\pi \rightarrow \pi^*$, ε_{307}–340 M^{-1}); IR (KBr) (stretching frequency): 3340 cm^{-1} (OH), 1260 cm^{-1} (glycosidic aryl alkyl C-O-C asymmetrical), 1050 cm^{-1} (glycosidic aryl alkyl C-O-C symmetrical), 1370 cm^{-1} (C=C), 2955 cm^{-1} (CH); MS (*m/z*) 305.04 $[M+Na]^+$; 2D HSQCT (DMSO-d_6) **C1α-arabinoside 11a**: ^{1}H NMR δ_{ppm} (500.13): **Ara**: 5.0 (H-1α), 3.61 (H-2α), 3.64 (H-4α), 3.51 (H-5α); **Thymol**: 6.55 (H-5), 2.86 (H-7); ^{13}C NMR δ_{ppm} (125 MHz): **Ara**: 95.9 (C1α), 69.7 (C2α), 77.4 (C3α); **Thymol**: 135.4 (C2), 131.4 (C4), 154.2 (C6), 35.9 (C7), 30.9 (C9), 30.9 (C10). **C1β-arabinoside 11b**: ^{1}H NMR δ_{ppm} (500.13): **Ara**: 4.90 (H-1β), 3.58 (H-2β), 3.60 (H-3β), 3.75 (H-4β), 3.52 (H-5β); ^{13}C NMR δ_{ppm} (125 MHz): **Ara**: 102.1 (C1β), 69.5 (C2β), 83.3 (C3β), 75.4 (C4β), 63.2 (C5β).

Statistical analysis

All the experiments were performed in triplicate and the data were calculated as means ± SD. A probability value of $P < 0.05$ was considered significant.

6.3. Results

The present glycosylation reaction was attempted under reflux using di-isopropyl ether at 68°C. However, the reaction mixture showed browning due to oxidation of thymol to thymoquinone. Hence, the reaction was carried out under $SCCO_2$ condition. In the present work, thymol **1** was reacted with carbohydrate molecules: D-glucose, D-galactose **2**, D-mannose **3**, D-fructose **4**, D-ribose **5**, D-arabinose **6**, maltose, lactose, sucrose, D-mannitol and D-sorbitol in presence of amyloglucosidase. But the enzymatic reaction occurred only with D-galactose **2**, D-mannose **3**, D-fructose **4**, D-ribose **5** and D-arabinose **6** (Fig. 6.5).

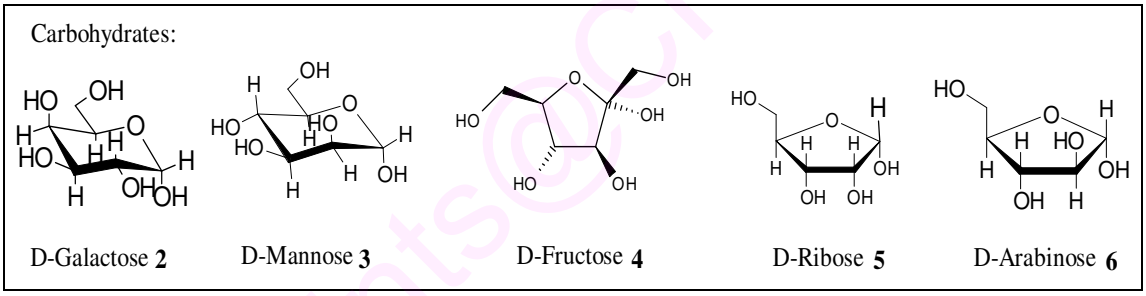
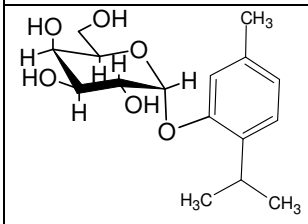
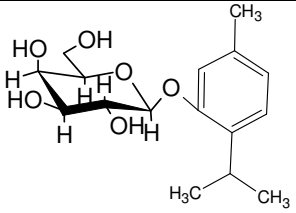
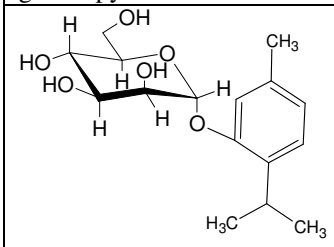
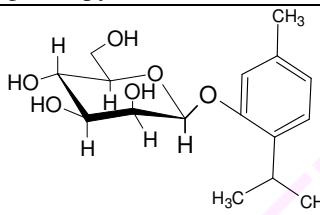
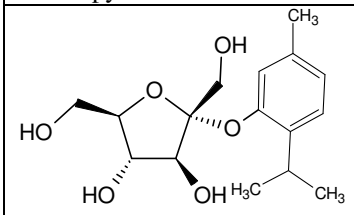
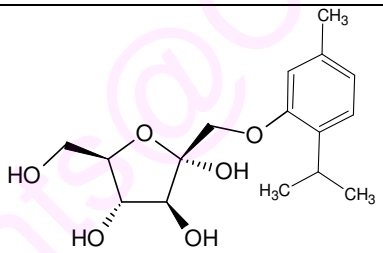
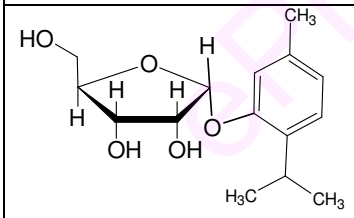
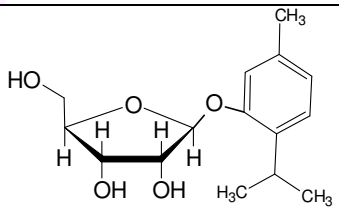
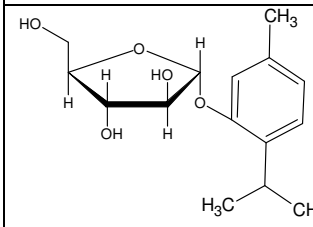
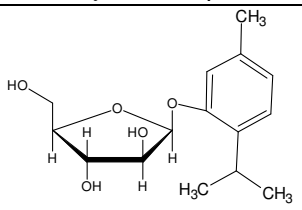


Fig. 6.5. Carbohydrates reacted with thymol.

The optimum conditions employed in this work were arrived at from several such optimization works involving a wide range of aglycon molecules in our laboratory. Optimum conditions employed for the reaction are 6.0 mmol of thymol with 6.0 mmol of carbohydrates **2–6**, in 10 ml of DMF in presence of amyloglucosidase (40% w/w carbohydrates corresponding to 680—810 μmol per min per mg of enzyme proportion in terms of hydrolytic activity units) and 3.0 ml of phosphate buffer (0.01 M, pH 6.0) under $SCCO_2$ conditions of 120 bar pressure at 50°C of temperature for 24 h (Fig. 6.1). The results from this glycosylation reaction are shown in Table 6.2. Thymol glycosides

formed were subjected to spectral characterization. Mass spectra also confirmed glycosides formation. The glycosides formed were found to be soluble in water. Thus, this study has shown that a complex phenol molecule like thymol could be glycosylated with diverse carbohydrate molecules using amyloglucosidase from *Rhizopus* mold under $SCCO_2$ conditions.

Table 6.2. Amyloglucosidase catalyzed synthesis of thymol glycosides in $SCCO_2$

Glycosides		Product (% Proportion)[a]	Yield (%)[b] NMR/ HPLC
 **7a**:Thymol-6-*O*-α-D-galactopyranoside	 **7b**:Thymol-6-*O*-β-D-galactopyranoside	**7a** (70.2) and **7b** (29.8) mono galactosides	29.7/15.4
 **8a**:Thymol-6-*O*-α-D-mannopyranoside	 **8b**:Thymol-6-*O*-β-D-mannopyranoside	**8a** (49.7) and **8b** (50.3) mono mannosides	23.5/27.6
 **9a**:Thymol-6-*O*-2-*O*-D-fructofuranoside	 **9b**:Thymol-6-*O*-1-*O*-D-fructofuranoside	**9a** (47.4) and **9b** (52.6) mono fructosides	54.2/34.1
 **10a**:Thymol-6-*O*-α-D-ribofuranoside	 **10b**: Thymol-6-*O*-β-D-ribofuranoside	**10a** (64.7) and **10b** (35.3) mono ribosides	20.6/18.2
 **11a**:Thymol-6-*O*-α-D-arabinofuranoside	 **11b**:Thymol-6-*O*-β-D-arabinofuranoside	**11a** (22.0) and **11b** (78.0) mono arabinosides	42.9/39.8

[a]NMR yields and product proportions were determined by 2D HSQCT NMR - H-1/C1 and or H-6a & b/C6 peak areas or their cross peaks. [b] HPLC yields were determined as mentioned in the text. The yields were an average from two independent experiments.

Spectral characterization

Thymol glycosides were characterized by UV, IR, Mass and 2D HSQCT spectra and were recorded along with ^{1}H and ^{13}C NMR spectra. Mass spectra with electron spray ionization (ESI) source were obtained as follows: MS (*m/z*) 311.93 $[M-1]^+$; 311.93 $[M-1]^+$; 312.80 $[M]^+$; 282.01 $[M]^+$ and 305.04 $[M+Na]^+$. UV spectra of the thymol glycosides, showed shifts in the $\pi \rightarrow \pi^*$ band in the 278–307 nm (276 nm for free thymol) range and IR spectra showed shifts in the OH stretching frequency of 3340 cm^{-1} — 3480 cm^{-1}, glycosidic aryl alkyl C-O-C asymmetrical at 1250 cm^{-1} — 1260 cm^{-1} and glycosidic aryl alkyl C-O-C symmetrical at 1050 cm^{-1} — 1070 cm^{-1}, C=C at 1370 cm^{-1} and CH at 2915 cm^{-1} — 2955 cm^{-1} range indicating that thymol had undergone glycosylation.

In the 2D HSQCT spectra of the thymol glycosides, the following glycoside formation were confirmed from their respective chemical shift values: from D-galactose **2** to C1α galactoside **7a**, at 95.5 ppm and H-1α at 4.97 ppm and C-1β-galactoside **7b** to C-1β at 101.9 ppm and H-1β at 4.91 ppm; from D-mannose **3** to C3α mannoside **8a** at 78.1 ppm and H3 α at 3.0 ppm and C-1β-mannoside **8b** to C-1β at 101.7 ppm and H-3β at 3.58 ppm; from D-fructose **4**, 2-*O*-D-fructofuranoside **9a** to C2 at 104.2 ppm and H-3 at 4.30 ppm and 1-*O*-D-fructofuranoside **9b** to C2 at 98.1 ppm and H-3 at 4.28 ppm; from D-ribose **5** to C1α riboside **10a** at 93.8 ppm and H-1α at 4.70 ppm and C-1β riboside **10b** to C-1β at 96.3 ppm and H-1β at 5.05 ppm; from D-arabinose **6** to C1α arabinoside **11a** to C1α at 95.9 ppm and H-1α at 5.0 ppm and C1β arabinoside **11b** to C1β at 102.1 ppm and H-1β at 4.90 ppm (Figs. 6.6 to 6.15). The Mass spectra also confirmed the formation of the above mentioned glycosides (Figs. 6.16 to 6.20).

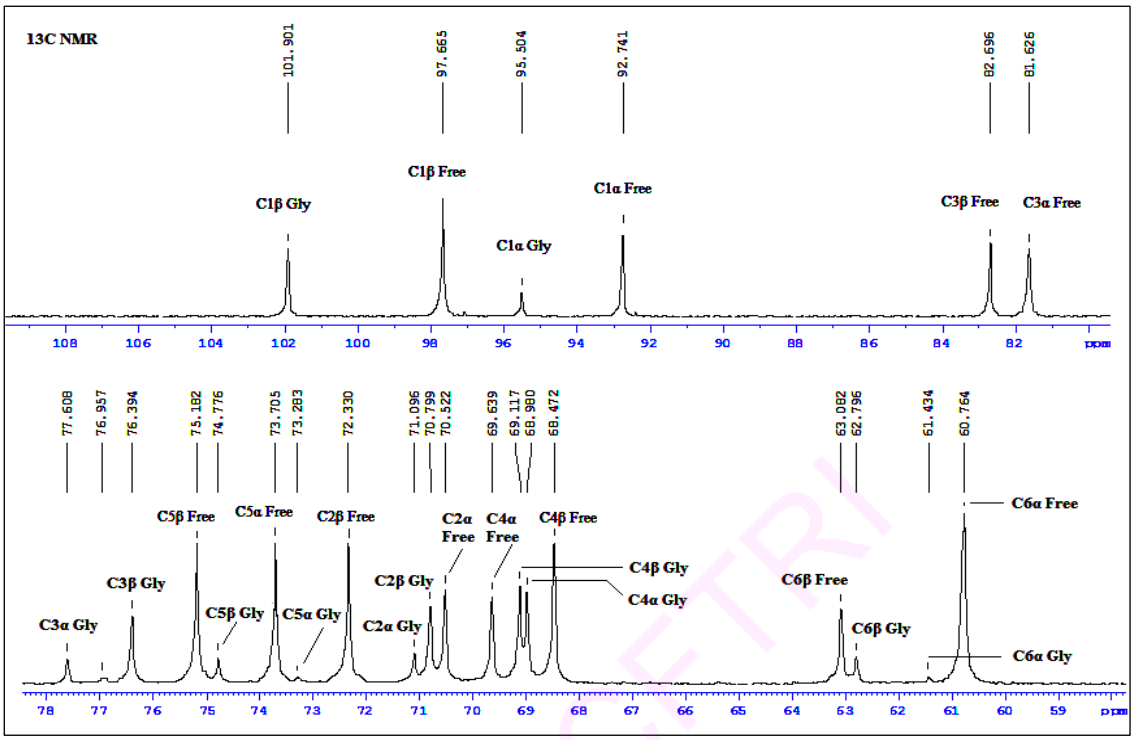


Fig. 6.6. ^{13}C NMR spectrum of thymol-6-*O*-D-galactopyranoside **7a-b.**

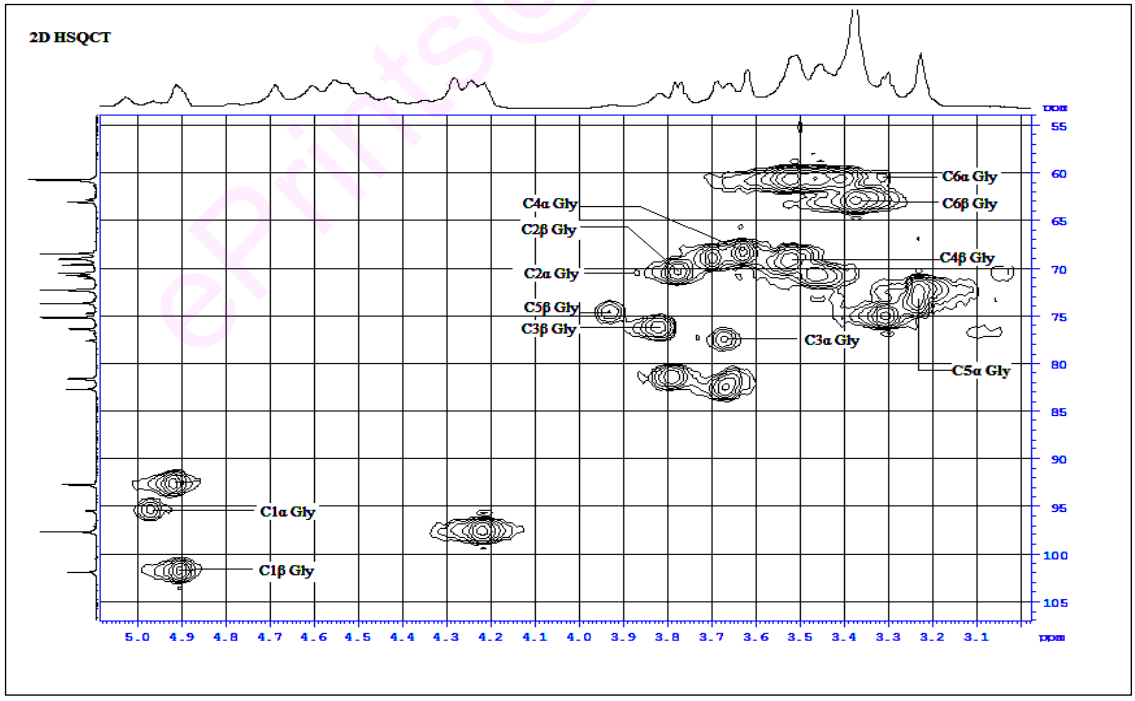


Fig. 6.7. 2D HSQCT spectrum of thymol-6-*O*-D-galactopyranoside **7a-b** showing the region between 3.0—5.1 ppm.

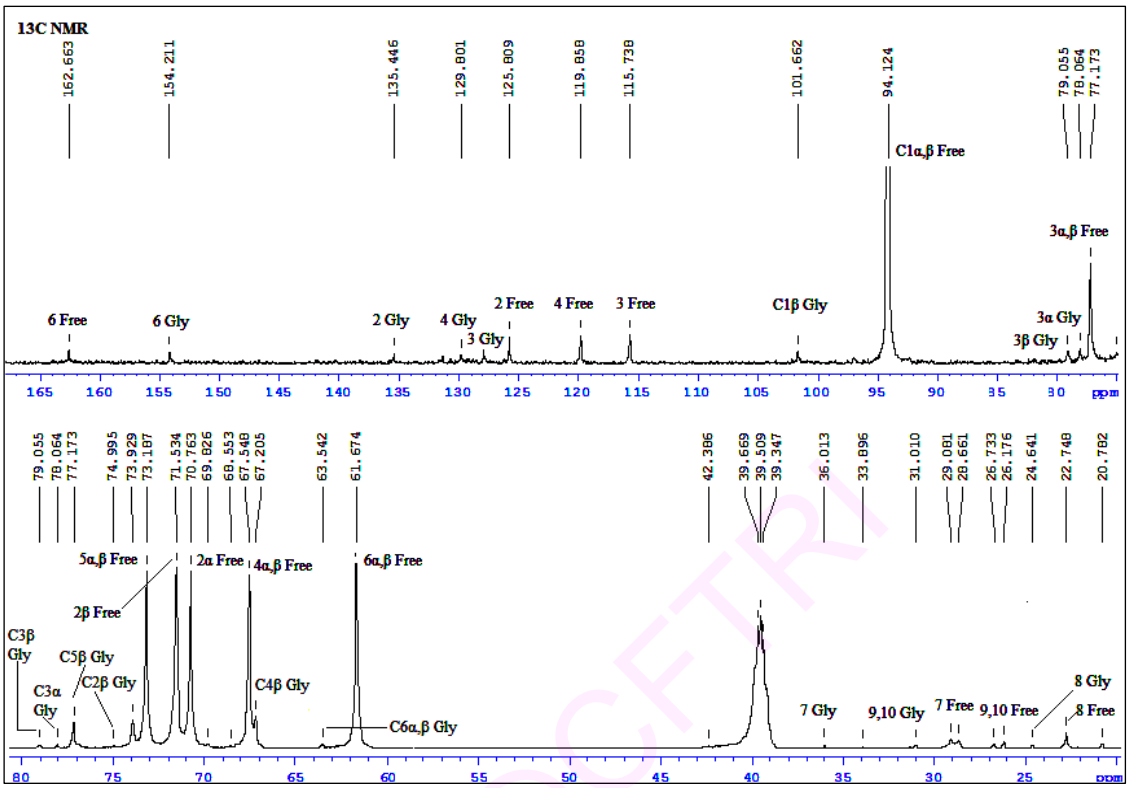


Fig. 6.8. ^{13}C NMR spectrum of thymol-6-*O*-D-mannopyranoside **8a-b**.

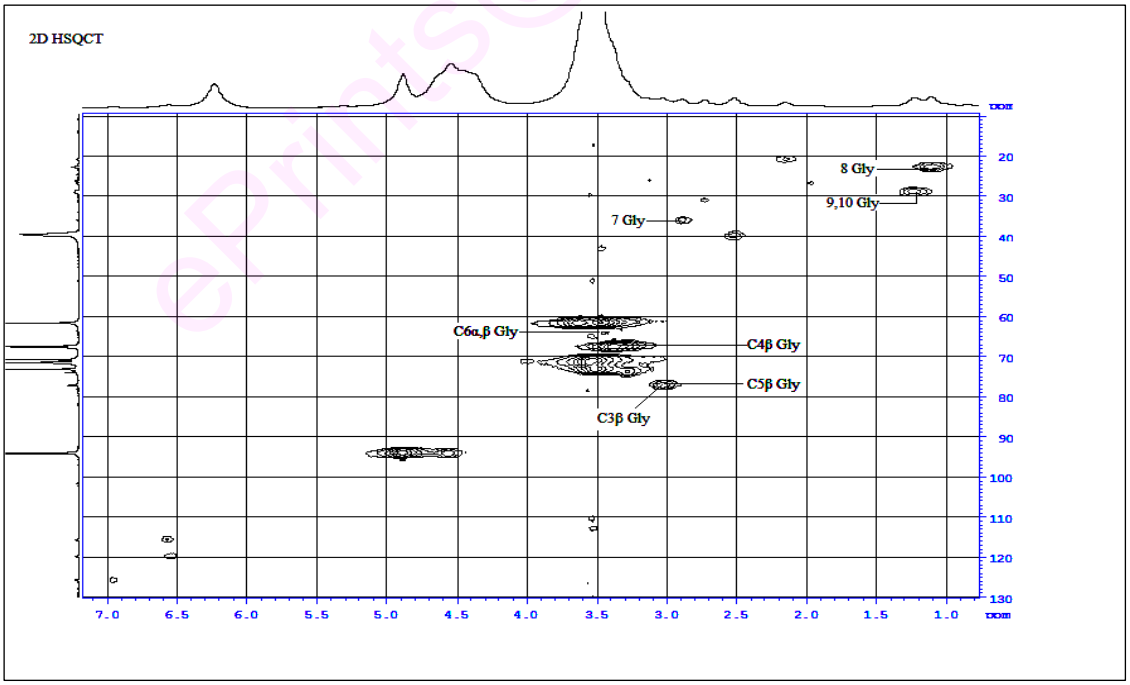


Fig. 6.9. 2D HSQCT spectrum of thymol-6-*O*-D-mannopyranoside **8a-b** showing the region between 0.75—7.25 ppm.

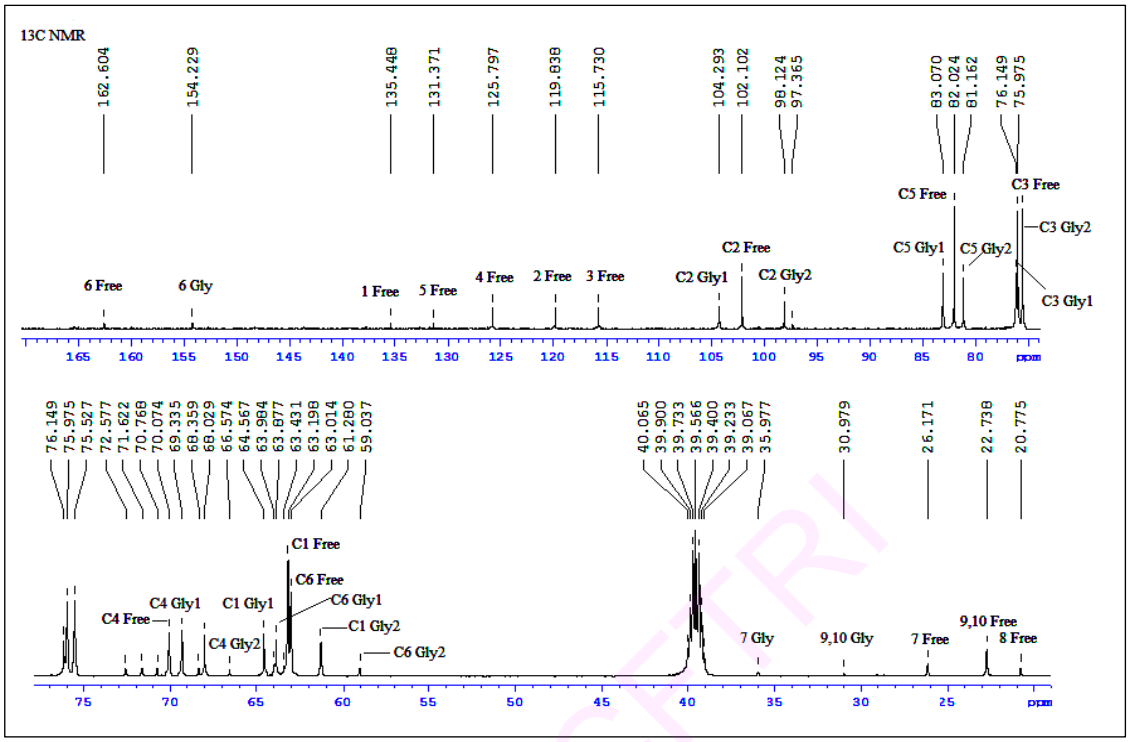


Fig. 6.10. ^{13}C NMR spectrum of thymol-6-*O*-D-fructofuranoside **9a-b**.
2-*O*-D-fructoside 9a: Gly 1.
1-*O*-D-fructoside 9b: Gly 2.

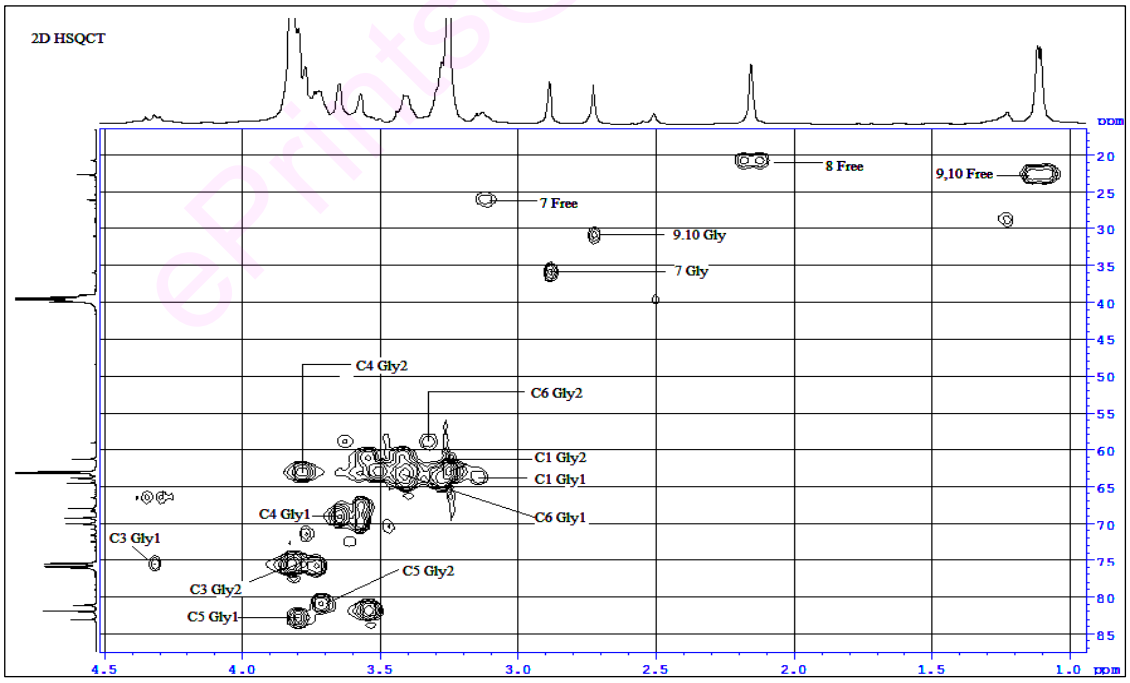


Fig. 6.11. 2D HSQCT spectrum of thymol-6-*O*-D-fructofuranoside **9a-b** showing the region between 0.9—4.5 ppm.

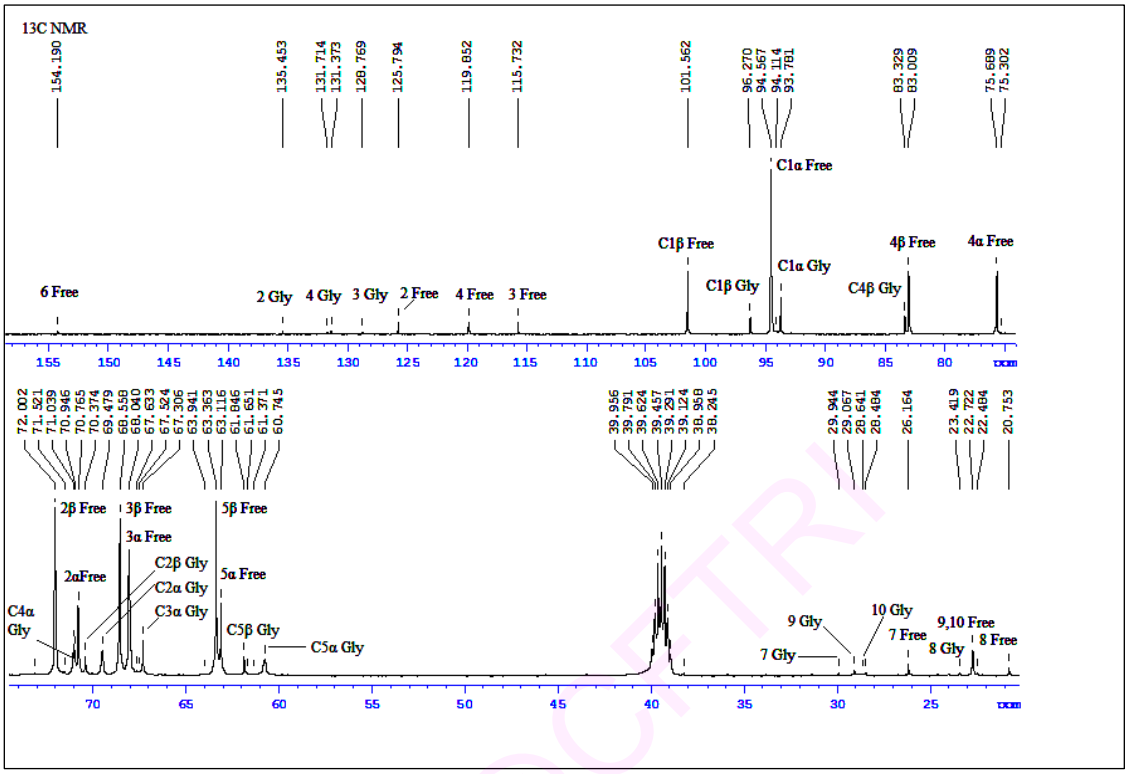


Fig. 6.12. ^{13}C NMR spectrum of thymol-6-*O*-D-ribofuranoside **10a-b**.

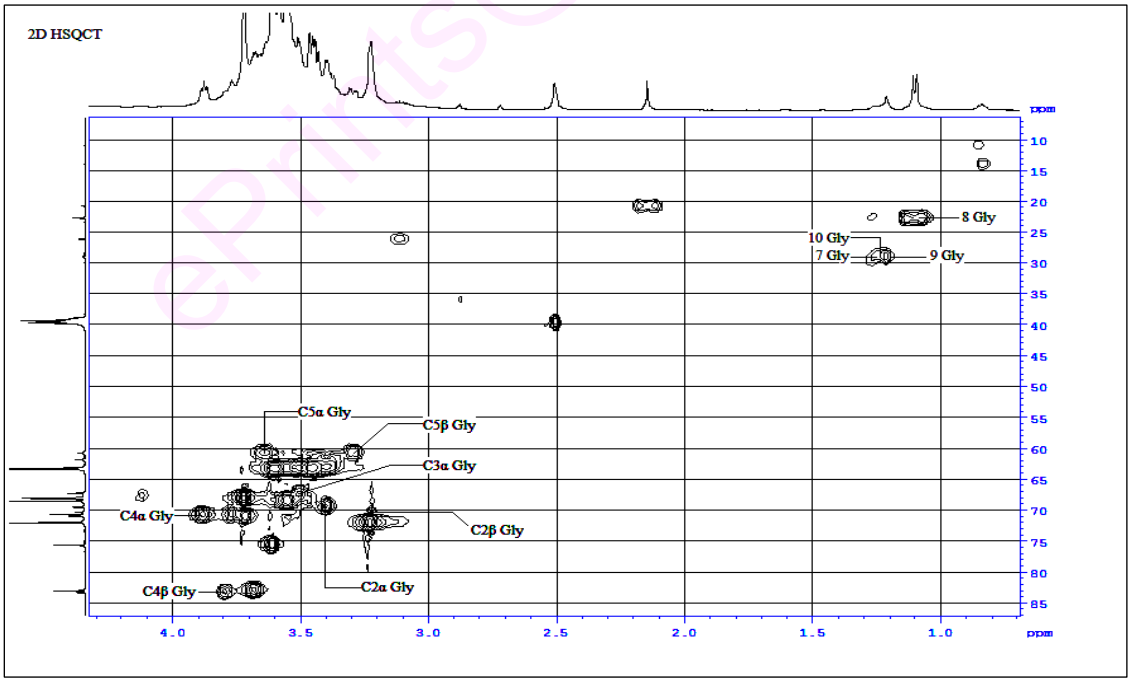


Fig. 6.13. 2D HSQCT spectrum of thymol-6-*O*-D-ribofuranoside **10a-b** showing the region between 0.5—4.5 ppm.

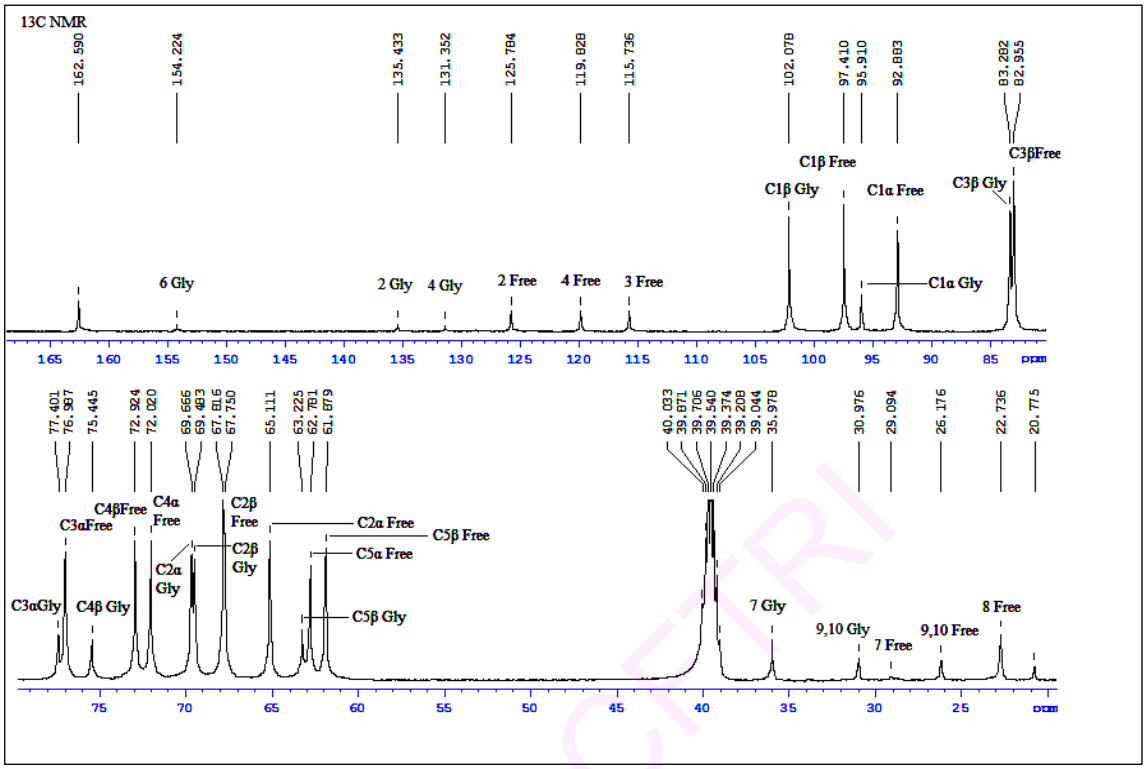


Fig. 6.14. ^{13}C NMR spectrum of thymol-6-*O*-D-arabinofuranoside **11a-b**.

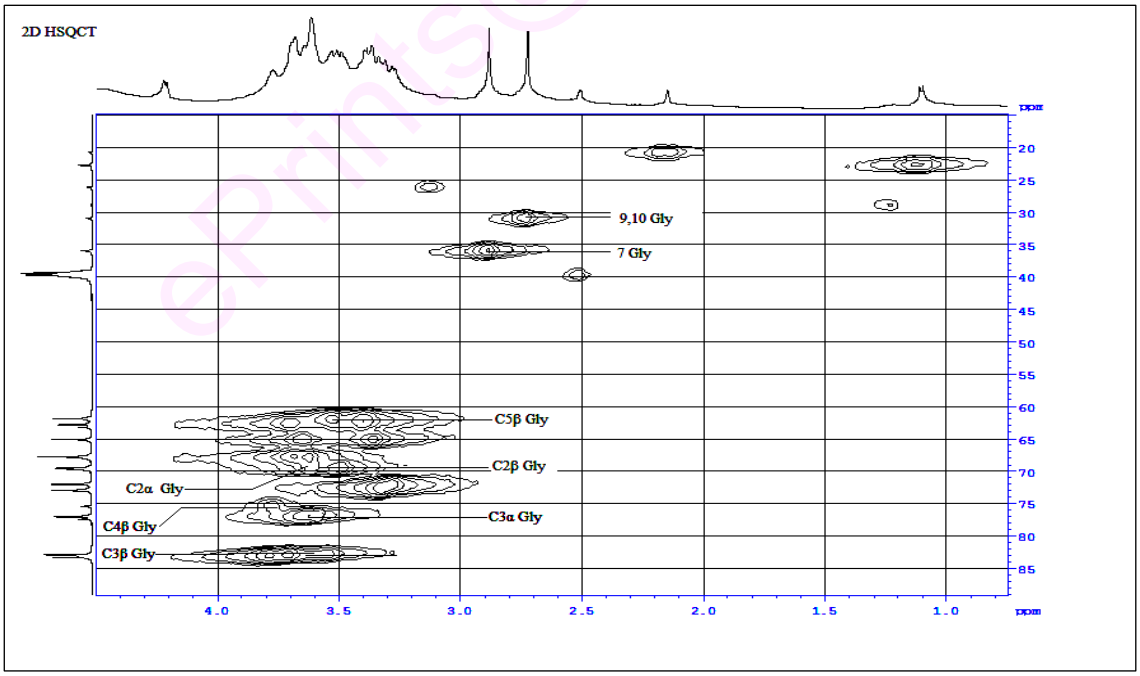


Fig. 6.15. 2D HSQCT spectrum of thymol-6-*O*-D-arabinofuranoside **11a-b** showing the region between 0.5—4.5 ppm.

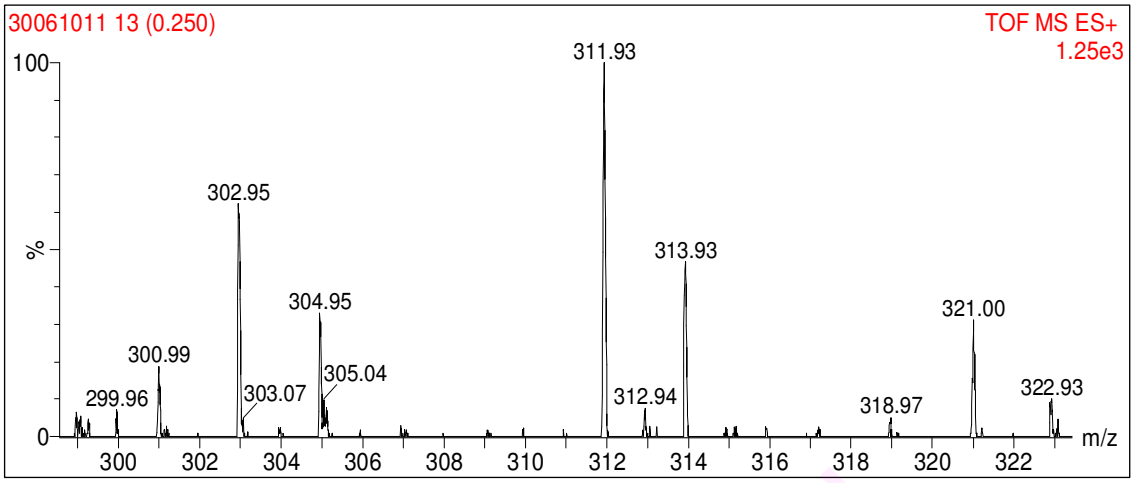


Fig. 6.16. Mass spectrum of thymol-6-*O*-D-galactopyranoside **7 a-b**.

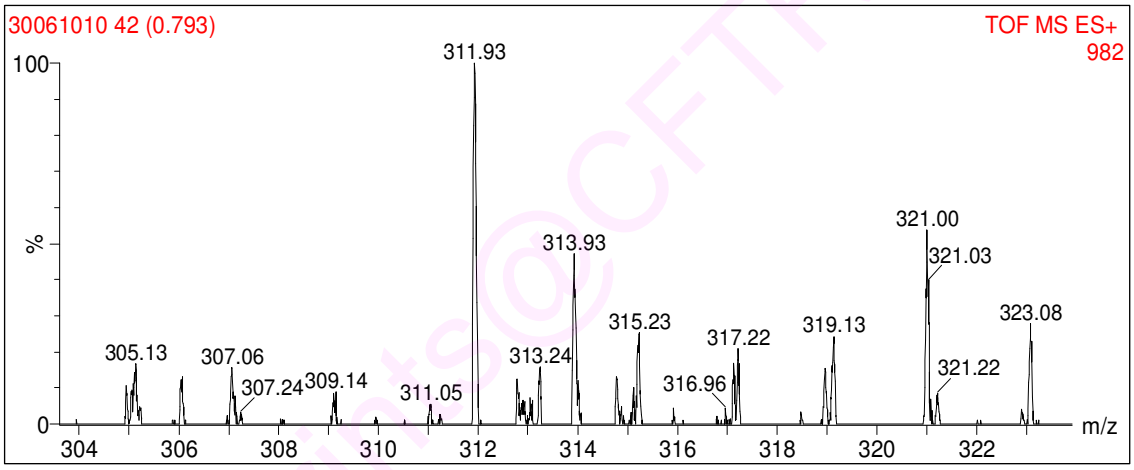


Fig. 6.17. Mass spectrum of thymol-6-*O*-D-mannopyranoside **8 a-b**.

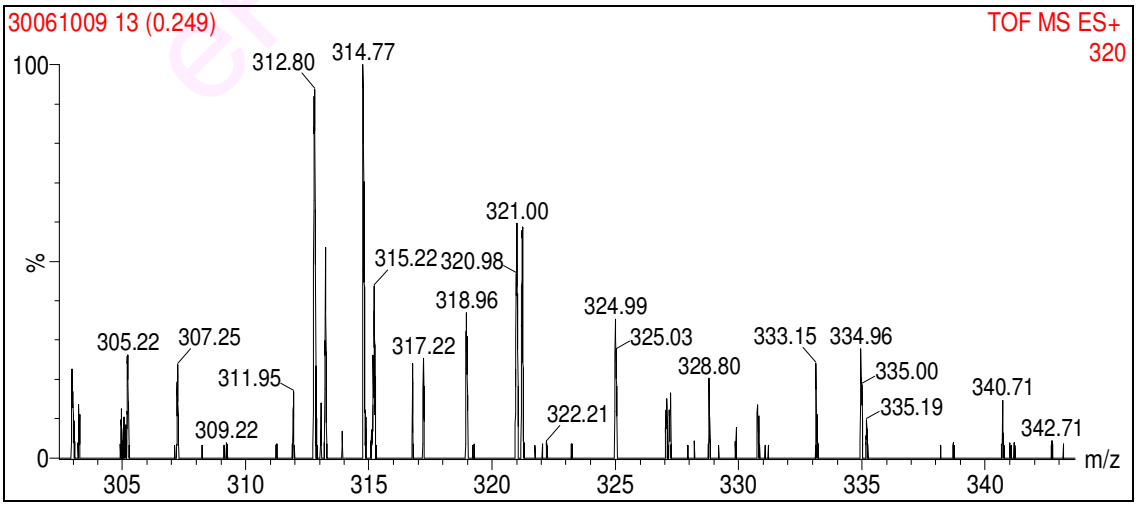


Fig. 6.18. Mass spectrum of thymol-6-*O*-D-fructofuranoside **9 a-b**.

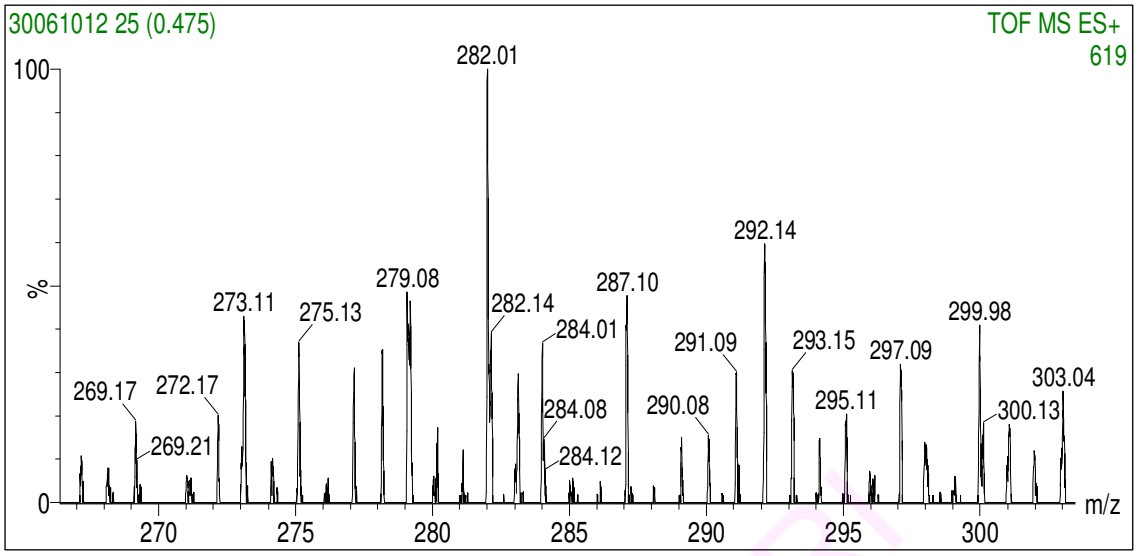


Fig. 6.19. Mass spectrum of thymol-6-*O*-D-ribofuranoside **10 a-b**.

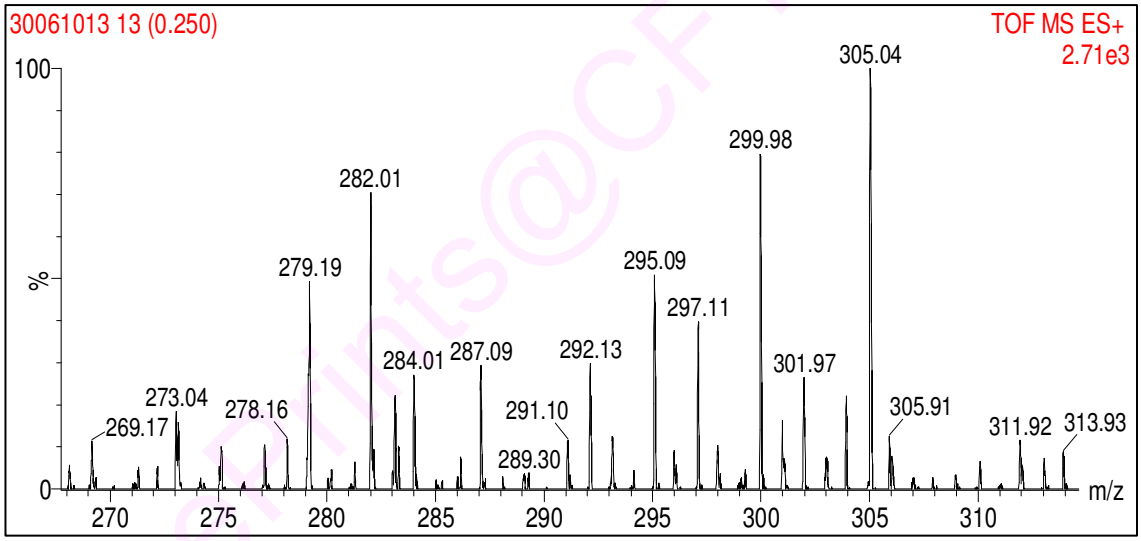


Fig. 6.20. Mass spectrum of thymol-6-*O*-D-arabinofuranoside **11a-b**.

Only mono glycosylated products were detected by 2D NMR. Thymol glycosides did not change colour on treatment with dilute alkali (1 ml of 0.1 M NaOH added to 10 mg glycoside in 1 ml of water) indicating that the phenolic OH groups is glycosylated. The NMR chemical shifts of C2, C3 and C4 carbons of thymol showed reaction at the free phenolic OH of C6 of thymol. Of the glycosides synthesized in the present work, thymol-6-*O*-D-galactopyranoside **7 a-b**, thymol-6-*O*-D-mannopyranoside **8 a-b**, thymol-6-*O*-2-*O* **9a** and thymol-6-*O*-1-*O*-fructofuranoside **9b**, thymol-6-*O*-D-ribofuranoside **10 a-b** and thymol-6-*O*-D-arabinofuranoside **11a-b** are reported for the first time.

6.4. Discussion

The glycosylation reaction did not occur with D-glucose. In several enzymatic reactions carried out in our laboratory, D-glucose has always reacted even under $SCCO_2$ conditions (Vijayakumar and Divakar 2005; Sivakumar and Divakar 2006; Charles *et al.* 2009). Apart from C-1 glycosylated and 2-*O* or 1-*O*-arylated products, no other secondary hydroxyl groups of the carbohydrates were found to react. Amyloglucosidase (EC 3.2.1.3) cleaves α (1, 4)-and α (1, 6)-glycosidic linkages from the non-reducing end of starch and related maltooligosaccharides to give glucose (Ashikari *et al.* 1986). Hence, the reaction was carried out under $SCCO_2$ condition.

In the present work, thymol was reacted with carbohydrate molecules-D-glucose, D-galactose, D-mannose, D-fructose, D-ribose, D-arabinose, maltose, lactose, sucrose, D-mannitol and D-sorbitol in presence of amyloglucosidase. This reaction did not occur in the absence of the above mentioned enzyme and absence of buffer (Sivakumar and Divakar 2006; McCarter and Withers 1994). Among the eleven carbohydrates employed in this present work, D-glucose is the most natural carbohydrate substrate for the enzyme amyloglucosidase and the other carbohydrate molecules like disaccharides-maltose, lactose, sucrose and the two sugar alcohols-D-mannitol and D-sorbitol also did not react. Earlier studies on synthesis of retinol glycosides under $SCCO_2$ conditions had shown that the more hydrophilic disaccharides and sugar alcohols did not either react or gave very low yields (Charles *et al.* 2009). Studies had also shown that differential bindings between the substrates (thymol and carbohydrates in this case) and the enzyme play a crucial role in determining whether the reaction occurs or not and the extent of the reaction (Vijayakumar and Divakar 2005; Sivakumar and Divakar 2006; Charles *et al.* 2009). Due to the fact that the competition in binding between thymol and the

carbohydrates for the active site of the enzyme which determines the transfer of the donor group to the acceptor molecule. But in this present study, thymol molecule could bind more tightly than the carbohydrates at the active site of the enzyme and hence could not be available for accepting the carbohydrate molecules (of D-glucose, lactose, maltose, sucrose, D-mannitol and D-sorbitol) leading to no condusive conformational orientation of enzyme and substrate complex and hence no reaction in this case. Evidences are there for glucosyltransferases in the cultures of *Eucalyptus perriniana* have the highest specificity for thymol and are able to convert thymol into higher water soluble glucosides (Shimoda *et al.* 2006). In the present enzymatic procedure also, thymol reacted with diverse carbohydrate molecules (D-galactose, D-mannose, D-fructose, D-ribose and D-arabinose) and gave better conversion into water soluble thymol glycosides involving a simpler isolation procedure. Even the presence of a bulky isopropyl group in this structurally complex acceptor phenol did not pose steric hindrance when the mono saccharide carbohydrate molecules are transferred to its phenolic OH group. In enzymatic reactions involving amyloglucosidase in the regular formed hydrolytic reaction, catalytic amounts of the enzyme is sufficient to affect regio-selective hydrolysis. However, in reverse glycosylation reaction, a catalytic amount of the enzyme was not sufficient to affect glycosylation. Larger amounts of the enzyme (40% w/w of the carbohydrates corresponding to 680—810 μmol per min per mg of enzyme proportion in terms of hydrolytic activity units) were required. Such a larger amount results in loss of regio-selectivity in such cases as the enzyme catalyses several non-specific reactions as well. The glycosides formed were found to be soluble in water. Thus, this study has shown that a complex phenol molecule like thymol could be glycosylated with diverse carbohydrate molecules using amyloglucosidase from *Rhizopus* mold under $SCCO_2$ conditions.

6.5. Conclusion

Water soluble thymol glycosides were synthesized by supercritical carbon dioxide ($SCCO_2$) using amyloglucosidase enzyme from *Rhizopus* mold. Glycosides were formed with D-galactose **2**, D-mannose **3**, D-fructose **4**, D-ribose **5** and D-arabinose **6**. The reaction occurred between the 1-*O*/2-*O* groups of D-fructose and C-1 group of D-galactose, D-mannose, D-ribose and D-arabinose with phenolic OH group of thymol resulting in monoglycosylated/arylated derivatives. D-glucose and the other carbohydrate molecules like disaccharides-maltose, lactose, sucrose and the two sugar alcohols-D-mannitol and D-sorbitol did not react. Thymol could be more tightly bound to the active sites of the enzyme than the above unreacted carbohydrate molecules resulting in a not-so-facile transfer of carbohydrate molecule to thymol.

Thus thymol glycosides synthesized by glycosylation under $SCCO_2$ are capable of enhancing the bioavailability of thymol for promoting food and pharmacological applications in the future.

SUMMARY

SUMMARY

Black cumin (*Nigella sativa* L.) seeds were subjected to $SCCO_2$ and conventional Soxtec™ extractions for higher extract yield and higher extracted thymoquinone. $SCCO_2$ extraction provided the thymoquinone (TQ) with better yield and superior quality. It was found that higher pressure and lower temperature with intermediate solvent to material ratio favours to increase the extract yield, however it decreased the TQ extraction, whereas intermediate pressure and lower temperature with higher solvent to material ratio favours to increase the TQ extraction and decreases the extract yield. TQ is heat labile, photo reactive and highly volatile. At optimized conditions of $SCCO_2$ extraction (pressure 120 bar, temperature 40°C and solvent to material ratio 45 kg/kg) the obtained $SCCO_2$-1 extract showed lower extract yield but with a higher TQ extracted (79.03%) as experimental along with higher TQ content. Whereas in conventional solvent based Soxtec™ extractions, the yields were high, but with a lesser TQ content. Hence, extracting TQ by conventional extraction techniques involving high temperature has often been a failure. It is thereby clearly established that the maximum level of valuable volatiles such as TQ enriched extract without photo reactivity, thermal degradation and decomposition is possible, only through the $SCCO_2$ technology and not by conventional solvent extractions. Hence, the $SCCO_2$ extracts containing higher percentage of TQ extracted and TQ content is taken up for the exploration of chemical composition and application oriented studies like antibacterial and antioxidant activities.

Chemical composition was explored by GC and GC-MS along with NMR spectroscopy. Forty seven different compounds were identified in the supercritical CO_2 ($SCCO_2$-1 & 2) and HD $SCCO_2$ (hydrodistillate of $SCCO_2$-2) extracts. Of these, the occurrences of 16 volatile compounds were reported for the first time in *Nigella*

sativa seeds. The essential oil with a better quality in terms of richness of quinones and a higher recovery of main compounds were achieved by HD $SCCO_2$ extract. The total extract of $SCCO_2$-2 was purified and subjected to characterization along with 2D HSQCT NMR spectroscopy to deduce and identify the pharmacologically active four major quinonic phenol compounds: thymoquinone (TQ), dithymoquinone (DTQ), thymohydroquinone (THQ) and thymol (THY) which confirmed the presence of bioactive profiles in the extract. The major bioactive compound was isolated and identified as thymoquinone from the total extract of $SCCO_2$-2.

Among the obtained extracts, $SCCO_2$-1 showed higher antibacterial activity along with higher TQ extracted and higher TQ content which was followed by Soxtec™ extracts. The isolated thymoquinone (TQ) also showed lower MIC and MBC values against food borne bacterial pathogens. TQ showed broad spectrum antibacterial activities which are comparable with that of Ciprofloxacin antibiotic. Hence, the isolated thymoquinone (TQ) from black cumin seed appears to be a promising as a potential natural antibacterial agent, which could replace the commercially available synthetic antibiotics. All these confirmed that TQ could be the antibacterial principle for *Nigella sativa* L. seed.

The optimized $SCCO_2$ extract ($SCCO_2$-1) also showed higher antioxidant activity along with higher total phenolics which was followed by Soxtec™ extracts. It showed higher TQ content (12.3 ± 0.24 %) along with higher amount of TPC (77.40 ± 0.61 mg GAE/g) and higher antioxidant activities (DPPH value of 84.23 ± 0.94 µM TE/g and a FRAP value of 0.73 ± 0.03 mM TE/g) indicating the optimal performance of the supercritical extract. Higher TQ content of the $SCCO_2$ extract directly correlated to the higher antioxidant activities. $SCCO_2$ extracts were found to have the least extractive yield but with higher TQ content than the Soxtec™ extracts. Isolated

TQ showed higher antioxidant properties with higher TPC than the synthetic antioxidants (BHA and α-tocopherol). The isolated thymoquinone (TQ) exhibited potent antioxidant activity which appears as a potential natural antioxidant agent which confirmed TQ could be the antioxidant principle for *Nigella sativa* L. seed. The bioactive thymoquinone (TQ) isolated from *Nigella sativa* seed, which could replace the commercially available synthetic antioxidants.

In order to facilitate further application oriented efforts, of the four quinonic phenol compounds the basic one thymol was taken up for enzymatic glycosylation for arriving at a most suitable bioactive form. For this water soluble thymol glycosides were synthesized using amyloglucosidase enzyme from *Rhizopus* mold by supercritical carbon dioxide ($SCCO_2$). The thymol glycosides synthesized by glycosylation under $SCCO_2$ were capable of enhancing the bioavailability of thymol for promoting food and pharmacological applications in the future. Water soluble glycosides were successfully formed with D-galactose, D-mannose, D-fructose, D-ribose and D-arabinose. The reaction occurred between the 1-*O*/2-*O* groups of D-fructose and C-1 group of D-galactose, D-mannose, D-ribose and D-arabinose with the phenolic OH group of thymol resulting in monoglycosylated/arylated derivatives. Thymol formed glycosides in yields ranging from 20.6 to 54.2%. Of the glycosides synthesized in the present work, thymol-6-*O*-D-galactopyranoside (α and β forms), thymol-6-*O*-D-mannopyranoside (α and β forms), thymol-6-*O*-2-*O*-D-fructofuranoside and thymol-6-*O*-1-*O*-D-fructofuranoside, thymol-6-*O*-D-ribofuranoside (α and β forms) and thymol-6-*O*-D-arabinofuranoside (α and β forms) are reported for the first time. Thus, the biotechnologically synthesized thymol glycosides are capable of enhancing the bioavailability of thymol for effective food and pharmacological applications in the future.

The bioactive thymoquinone (TQ) obtained (from *Nigella sativa* seeds) through the supercritical mode could serve as a biotechnological tool for promoting it as a potential as well as a natural antibacterial and antioxidant agent. Hence, synthetic antibiotics and antioxidants could be replaced by the major bioactive compound: Thymoquinone.

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PUBLICATIONS

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