Characterization of bioactive components from Chiba seed (*Psoralea corylifolia L.*) extracts obtained by supercritical carbon dioxide

A thesis submitted to the

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Doctor of Philosophy

in

BIOTECHNOLOGY

by

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Declaration

I hereby declare that the thesis entitled "Characterization of bioactive components from Chiba seed (*Psoralea corylifolia* L.) extracts obtained by supercritical carbon dioxide" submitted for the degree of Doctor of Philosophy in Biotechnology to the University of Mysore is the result of the work carried out by me under the guidance of Dr. K. Udaya Sankar, Scientist, Department of Food Engineering, Central Food Technological Research Institute, Mysore during the period 2006-2010.

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

Date:

Place: Mysore (B. Manohar)

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Certificate

I, B. MANOHAR, certify that this thesis is the result of research work done by me under the supervision of Dr. K. Udaya Sankar at Department of Food Engineering, Central Food Technological Research Institute, Mysore. 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 or diploma of this or any other University.

Signature of Doctoral candidate

Signed by me on

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Date:

Date:
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Institution with name and official seal

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ABSTRACT

The rapid emergence of nutraceuticals and functional foods prepared from plant extracts has gained enormous importance in recent times due to natural products providing several advantages such as fewer adverse health effects, better patient tolerance, relatively low price and acceptance due to a long history of use. Among the various plant materials available for extraction of bioactives, the present thesis focuses on *Psoralea corylifolia* L.. The main objectives of the thesis are: a) Preparation of plant extract from *P. corylifolia* seeds that contain important bioactives like bakuchiol and psoralen using supercritical carbon dioxide ($SCCO_2$) extraction technology. b) Characterization of the extract in terms of important bioactives. c) Enrichment of the extract with higher concentration of bioactives by adopting high vacuum molecular distillation technology d) Enzymatic synthesis of water soluble bakuchiol glycosides and study of their anti-oxidant and angiotensin converting enzyme **(**ACE) inhibitory activities.

P. corylifolia having high medicinal values are widely used in Chinese and Indian Ayurvedic medicinal systems. It is widely used in traditional medicine to treat a variety of diseases and possesses anti-tumor, anti-bacterial, anti-helmenthic, anti-oxidant and cytotoxic properties. The bakuchiol from *P.corylifolia* is thermally labile and forms cyclo-bakuchiol due to heat in acidic conditions. Hence, the extraction and purification of bioactives from *P.corylifolia* needs mild processing conditions. The present study reports, for the first time, extraction of bioactives from *P. corylifolia* seed using supercritical CO_2 extraction which has proven itself as an alternative processing technology to organic solvent extraction. Critical properties like critical temperature, critical pressure, critical volume which are important for solubility determination, process design and development were predicted for key components bakuchiol and psoralen, based on four different group contribution methods such as Ambrose, Joback, Klincewicz-Reid and Lydersen methods. Prediction of vapor pressure of key components showed that there is substantial difference between the vapor pressure of bakuchiol and psoralen.

The extraction of bioactives from *P.corylifolia* seeds was carried out using the high pressure $SCCO_2$ system at pressures 220, 260 and 300 bar and at temperature of 40°C. The mass transfer of the extraction was studied based on Diffusion, Exponential, Langmuir and Logistic models and comparative evaluation of the

models was carried out. LC-MS chromatogram of the extract identified presence of nine compounds. The studies established that the extract having bakuchiol concentration of 51% was possible with $SCCO_2$ extraction and was substantially higher than of solvent extraction process. The solubility of bakuchiol and psoralen at various pressures was predicted based on equation of state models Peng-Robinson and Soave-Redlich-Kwong. The models indicated high enhancement factors for both the key components implying high non-ideal behavior.

Molecular distillation technique was applied to increase the bakuchiol content in the extract obtained by $SCCO_2$. The effect of process variables such as vacuum, temperature and wiper speed on bakuchiol concentration was studied based on Response Surface Methodology following Box-Behnken design of experiment. The predicted second order polynomial model was solved for the maximum bakuchiol content in the distillate. A predicted maximum of 72% bakuchiol was possible under the optimized process conditions and the same was confirmed by the experiment. Further, it was observed that both principal components bakuchiol and psoralen co-distill during the molecular distillation. The study established that the separation and concentration of bakuchiol from the extract was possible using molecular distillation technique without degradation / cyclisation of bakuchiol.

Bakuchiol exhibits poor water solubility, stability and absorbability. Glycosylation improves the pharmacological property by increasing the water solubility of bakuchiol. The reactions using amyloglucosidase from *Rhizopus* mold were carried out in two different media: one by conventional reflux method and the other under supercritical CO_2 atmospheric conditions. Out of eleven carbohydrate molecules selected for the reaction, D-glucose, D-ribose and D-arabinose gave glycosides in yields of 9.0% to 51.4% under conventional reflux method. Under supercritical CO_2 atmosphere (100 bar pressure at 50°C), bakuchiol formed glycosides with D-glucose, D-galactose, D-mannose, D-fructose, D-ribose, D-arabinose, D-sorbitol and D-mannitol in yield range 9% to 46.6%. Bakuchiol glucosides showed reasonable antioxidant and ACE inhibitory activity compared to BHA.

Thus the investigations clearly brought out the extractability of bioactives from *P. corylifolia* using $SCCO_2$, enrichment using molecular distillation and preparation of water soluble glycosides to enhance the bioavailability and bioactivity of the bioactive components of *P. corylifolia*.

CONTENTS **Page No.**

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
δ	Chemical shift value
µg	Microgram
µL	Microlitre
[α]	Optical rotation
A	Absorbance
Å	Angstrom
ACE	Angiotensin converting enzyme
ANOVA	Analysis of variance
AARD	Average absolute relative deviation
ARD	Absolute relative deviation
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CAGR	Compounded annual growth rate
CFIA	Canadian Food Inspection Agency
cm	Centimeter
CO_2	Carbon dioxide
CSIR	Council of Scientific and Industrial Research
DMF	Dimethyl formamide
DMSO-d_6	Deuteriated Dimethyl sulfoxide
DPPH	2,2 diphenyl-1-picryl hydrazyl
EEC	European Economic Commission
Ev	Electronvolt
FAO	Food and Agricultural organization
FDA	Food and Drug administration (of USA)
g	Gram
GRAS	Generally regarded as safe
h	Hour
HPLC	High Performance Liquid Chromatography
HSQCT	Heteronuclear Single Quantum Coherence Transfer
Hz	Hertz
IC_{50}	Half maximal inhibitory concentration
IR	Infra red
J	Coupling constant
K	Degree kelvin
kg	Kilogram

Kv	Kilovolts
M	Molarity
MCA	methylcholanthrene
mg	Milligram
MHz	Mega hertz
min	Minute
ml.	Milliliter
mm	Millimeter
mmol	Millimole
mol	Mole
MS	Mass spectroscopy
N	Normality
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NSF	National Science Foundation
^{o}C	Degree centigrade
ppm	Parts per million
PR	Peng-Robinson
PUFA	Polyunsaturated Fatty Acids
rpm	Revolutions per minute
RSM	Response Surface Methodology
RT	Retention time
$SCCO_2$	Supercritical carbon dioxide
SCF	Supercritical fluid
SCFE	Supercritical fluid extraction
sec	Seconds
SRK	Soave-Redlich-Kwong
TLC	Thin layer chromatography
UV	Ultra violet
v/v	Volume by volume
v/w	Volume by weight
w/v	Weight by volume
w/w	Weight by weight
WHO	World health organization
^{1}H	Proton
2D	Two-Dimensional
^{13}C	Carbon-13

CHAPTER 1

Introduction

1.0. Nutraceuticals

Nutraceutical refers to extracts of foods claimed to have medicinal effect on human health. Nutraceuticals are a large and diverse class of compounds, which occur naturally in plants. Although, these compounds are considered non-nutritive, many of them possess antioxidant, antimutagenic, anti-oestrogenic, anti-carcinogenic and anti-inflammatory properties that might potentially be beneficial in preventing diseases and protecting the stability of the genome. The term "nutraceutical" was coined from "nutrition" and "pharmaceutical" in 1989 by Dr. Stephen DeFelice [source: American Nutraceutical Association]. According to DeFelice, a nutraceutical is any substance that is a food or a part of a food and provides medical or health benefits, including the prevention and treatment of disease. Such products may range from isolated nutrients, dietary supplements and specific diets to genetically engineered designer foods, herbal products, and processed foods such as cereals, soups and beverages. In the United States, the term nutraceutical is commonly used in marketing and has no regulatory definition. One of the most important growing trends in food industry today is the demand for all natural food ingredients free of chemical additives. Increase in health awareness caused major changes in food, pharmaceutical and nutraceuticals industries. The nutraceutical market has grown multifold both in the developed and developing countries. People are becoming more concerned about their well being and are shifting to nutraceutical products to prevent the onset of any chronic diseases like diabetes, heart ailments, hypertension and weight gain.

According to one estimate, Indian nutraceutical market in 2007 was Rs 18.75 billion and expected to grow at 20 per cent compounded annual growth rate (CAGR) to achieve a market size of Rs 27 billion in 2009 [www.fnbnews.com]. The global market size of nutraceuticals is about $250 billion [Defelice, 1995]. A report on world nutraceuticals by M/s Freedonia Group Inc., USA states global demand for nutraceutical ingredients will grow at 5.8% annually through 2010 [www.marketresearch.com]. Best prospects include probiotics, soy additives, lycopene, lutein, sterol-based additives, green tea, glucosamine and chondroitin, and coenzyme Q10. China and India will be the fastest growing markets, while the US will remain the largest. Thus, plant-based therapeutic agents continue to have scientific, social, and commercial significance and appear to be gathering a momentum in health-relevant areas [Dev, 1999].

The rapid emergence of nutraceuticals has created a new trend in food processing industries. They are gaining popularity because of several advantages such as fewer side effects, better patient tolerance, relatively low price and acceptance due to a long history of use. The more important cause is that natural products provide rational means for the treatment of many diseases that are obstinate and incurable. The nutraceutical products are normally prepared from the extracts of purified bioactive components. The present thesis focuses on one of the highly valuable plants *Psoralea corylifolia* L. as the source for active biocomponents.

1.1. *Psoralea corylifolia* L.

The medicinal plant is distributed from China, India to Southeast Asia. Figure 1.1 shows the plant and seeds of *Psoralea corylifolia.* Table 1.1 lists botanical names of the plant along with common names in various languages around the world. It is reported in Indian traditional medicine that the seed, root and leaves of the plant are used in the form of powder to treat skin diseases, vitiligo etc. [Nadkarni, 1954; Chopra *et al.*, 1986; Khare, 2007]. Ancient Hindu physicians have made mention of its uses and according to some, it is a laxative, stimulant and aphrodisiac. *Psoralea corylifolia* seeds are extensively used in stomachic, deobstruent, anthelmintic, diuretic and also against certain skin diseases, like, leucoderma and leprosy [Chopra and Chatterjee, 1927; Gaind *et al*., 1965; CSIR- India, Annual report 2002-03]. According to the Chinese Pharmacopoeia, it has been used as a medicinal herb for warming kidney and activating yang, promoting inspiration and checking diarrhea [National Commission of Chinese Pharmacopoeia, 2005].

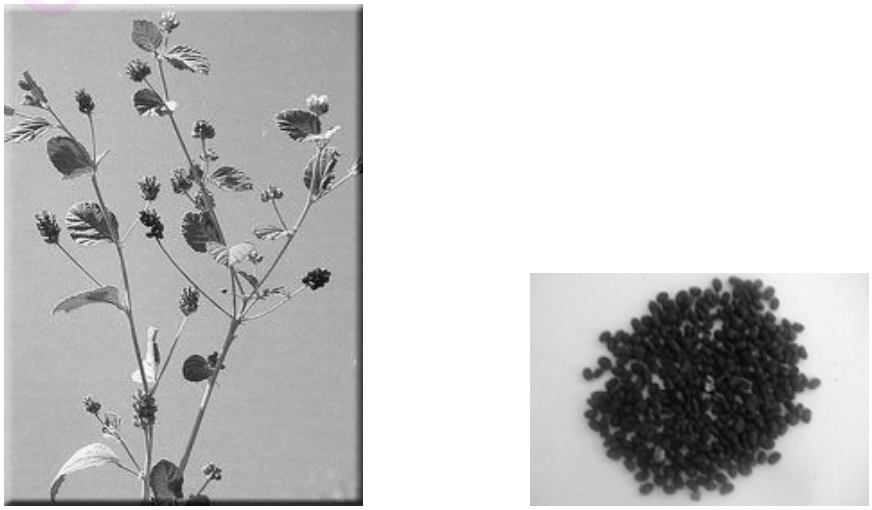


Fig. 1.1. The plant and seeds of *Psoralea corylifolia.*

It has also been reported that the plant bioactives offer a cure against certain skin diseases e.g. leucoderma and leprosy [Kondo *et al.,* 1990; Kotiyal and Sharma, 1992; Khan *et al*., 2009]. The seed extract of *P.corylifolia* is also suggested to be a useful remedy for bone fracture, osteomalacia and osteoporosis [Lim *et al*., 2009; Miura *et al.*, 1996].

Table 1.1. Common and popular names of *Psoralea corylifolia* around the world

Botanical name	:	*Psoralea pinnata*. L. or *Psoralea corylifolia* L. (*Psoralea corylifolia* Linn.)
Family genus	:	*Fabaceae, Papilionaceae*
English name	:	Babchi
Latin name	:	*Psoralea corylifolia*
Habitat	:	Common all over China and India in dry and sandy places
Arabic	:	Loelab el abid, Mahalep
Bengali	:	Barachi, Bavachi, Hakuch, Latakasturi
Chinese	:	Ku Tzu, Pu Ku Chih
Gujarati	:	Babchi, Bavacha, Bawachi
Hindi	:	Babachi, Babchi, Bavanchi, Bukchi
Kannada	:	Bavanchi
Marathi	:	Bavachya, Babchi, Bavachi
Persian	:	Waghchi
Sanskrit	:	Aindavi, Asitatvacha, Avalguja, Bakuchi, Chandralekha, Chandra prabha, Chandraraji, Kalameshi, Kambojii, Kantaka, Krishna, Krishnaphala, Sita, Sitavari, Soma, Somaraji.
Sinhalese	:	Bodi
Tamil	:	Karpokarishi, Karpurvarishi
Telugu	:	Bhavanji, Kalanginja, Karubogi, Korjastham
Urdu	:	Babechi

Source: Khare, 2007; www. frlht.org

1.1.1. Phytochemicals in *Psoralea corylifolia*

Psoralea corylifolia L. has been the subject of several investigations since 1890s [Dymock *et al.,* 1893]. The seeds are kidney shaped, 2-4 mm long, 2-3 mm broad and 1-15 mm thick, smooth, exalbuminous with straw colored hard testa. The seed contains volatile oil, coumarin, flavones, monoterpene phenols, lipid compounds, resins and stigmasteroids. The volatile oils include limonene, terpin-4-ol, linalool, β -caryophyllene and geranyl acetate. Coumarin derivatives include psoralen, isopsoralen, psoralidin, isopsoralidin, corylidin, bavacoumestan A, bavacoumestan, B sophoracoumestan A and 8-methoxypsoralen. Flavones include coryfolin, corylifolinin, bavachinin, isobavachin, neobavaisoflavone, bavachromene, corylin, bavachalcone and neobavachalcone. Monoterpene phenol includes bakuchiol. Lipids include triglycerides, diglycerides and monoglycerides. Others include free fatty acids, stigmasterol, daucosterol, triacontane, glucose and saponin. [http://www.shen-nong.com/eng/herbal/buguzhi.html]. The fatty acids obtained from the oil are found to be primarily palmitic, oleic and linoleic acids together with small amounts of stearic, lignoceric and linolenic acids. The pharmacologically active oil is identical with the unsaponifiable oil isolated by earlier workers [Gaind *et al*., 1965; Gupta *et al.*, 1962].

Since the seed was first chemically examined, a number of constituents (more than 188) have been detected so far [Chen *et al*., 2005]. The seeds are reported to contain an array of compounds belonging to different chemical groups such as furanocoumarins, coumestrol group, chalcones and flavones [Bhalla *et al*., 1968; Chakrovarti *et al*., 1948; Chen *et al*., 1996; Satyavathi *et al.*, 1987]. The active compounds identified from the herb include coumarins, benzofuran glycosides, flavonoids and meroterpene, such as psoralenoside, isopsoralenoside, psoralen, isopsoralen, neobavaisoflavone, bavachin, psoralidin, isobavachalcone, corylifol-A and bakuchiol, etc. [Agarwal *et al*., 2006; Hsu *et al*., 2001; Qiao *et al*., 2006; Ruan *et al*., 2007; Yin *et al.*, 2006; Yu *et al*., 2005]. The principal active chemical compounds in *Psoralea corylifolia* are: bakuchiol and psoralen [Dev, 1999]. A simple and efficient method of analysis for ten compounds of *Psoralea corylifolia* L. has been described [Wang *et al*., 2009].

1.1.1.1. Psoralen: Psoralen is the fundamental linear furocoumarin, the most important active principle of the seeds of *Psoralea corylifolia* [Anand *et al*., 1978; Innocenti *et al.,* 1977; Rangari and Agarwal, 1992]. Chemical and pharmacological investigations led to the isolation of one of the active components of the chiba seed psoralen [Jois *et al.,* 1933]. It is structurally related to coumarin by the addition of a fused furan ring, and may be considered as a derivative of umbelliferone. Psoralen occurs naturally in the seeds of *Psoralea corylifolia*, as well as in the common Fig (*Ficus carica*) and West Indian satinwood (*Zanthoxylum*). Although safe to mammals, it should be used with care since many furocoumarins are extremely toxic to fish, and some are indeed used in streams in Indonesia to catch fish. The chemical structure is shown in Figure 1.2.

CAS Number: 66-97-7

IUPAC name: 7H-furo [3, 2-g] chromen-7-one

Chemical formula: $C_{11}H_6O_3$

Molecular mass: 186.16 g/mol

Melting point: 158-161 °C

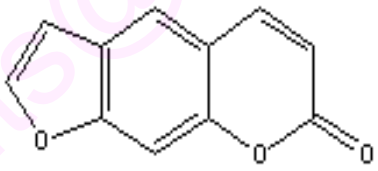


Fig. 1.2. Chemical structure of psoralen

1.1.1.2. Isopsoralen: Isopsoralen is a structural isomer of psoralen, and it was found to be identical with angelicin [Jois *et al*., 1933]. Angelicin (isopsoralen) is a photosensitizing agent and used for determining DNA/RNA structures in cell and microorganisms [Kittler *et al.,* 1980]. Angelicin and its derivatives occur in a number of plants belonging to the *Umbelliferae* family. The chemical structure is shown in Figure 1.3. The compounds have been tested clinically in combination with ultraviolet A radiation for use in the treatment of psoriasis. Angelicin, in the presence of ultraviolet A radiation, bound covalently to isolated DNA and to DNA in bacteria, yeast and cultured mammalian cells [Natarajan *et al.,* 1981].

CAS Number: 59-30-3

IUPAC name: 2H- furo[2,3-h]chromen-2-one

Chemical formula: $C_{11}H_6O_3$

Molecular mass: 186.17 g/mol

Melting point: 122.36°C

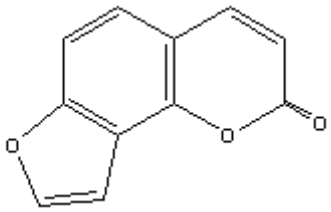


Fig. 1.3. **Chemical structure of isopsoralen**

1.1.1.3. Bakuchiol: The active compound, bakuchiol, has been obtained in a pure state and has been found to be a novel monoterpene phenol and named after the Sanskrit name of the plant [Mehta *et al*., 1973]. It has been shown to possess potent antibacterial activity [Satyavati, 1987]. The chemical structure is shown in Figure 1.4.

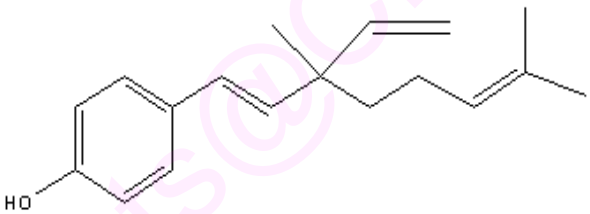


Fig. 1.4. Chemical structure of bakuchiol

CAS Number: 10309-37-2

IUPAC name: 4–(3,7–dimethyl–3–vinyl-octa–1,6–dienyl)phenol

Chemical formula: $C_{18}H_{24}O$

Molecular mass: 256.38 g/mol

Boiling point: 145-147^{o}C @ 0.7mm.Hg.

Bakuchiol has a hydroxyl group (IR: 3350, 1245cm^{-1}; 3,5-dinitribenzoate, melting point, 135-136°C), an aromatic ring (IR:1530cm^{-1}; PMR, vide infra), and olefinic linkages. The OH group is clearly phenolic. Bakuchiol contains three olefinic linkages and being $C_{18}H_{23}OH$ and aromatic ring and one of the olefinic linkages should be conjugated with the aromatic ring.

1.1.2. Biological activities of *Psoralea corylifolia* components

The *Psoralea corylifolia* plant has received much attention because of the

recent research findings, since the year 2000, establishing various biological activities for the plant's bioactive components. Table 1.2 lists the various biological activities reported with relevant literature reference. Furthermore, bakuchiol protects human red blood cells against oxidative haemolysis. Bakuchiol and other phenolic compounds in *Psoralea corylifolia* are shown to be effective in protecting biological membranes against various oxidative stresses [Haraguchi *et al*., 2002]. Extract from *P.corylifolia* has been studied as natural source for selective estrogen-receptor modulators [Xin *et al*., 2009]. Apart from the above, Dr. Dukes's online database on phytochemicals attribute biological activities such as anticarcinogic, antiflu, antigingitivitic, antimutagenic, antitubercular, antiviral, cytotoxicity to bakuchiol and psoralen, the main bioactives present in *Psoralea corylifolia* [Duke, 2009].

Table 1.2. Biological activities attributed to *Psoralea corylifolia.*

Biological activity	Literature reference
Anticancerous	Latha, 1996; Latha and Panikkar, 1998.
Antidermatophytic	Rajendraprasad *et al*., 2004.
Antiflarial	Qamaruddin *et al*., 2002.
Antifungal activity	Grover and Rao, 1979; Gupta *et al.*, 1962; Sharma and Singh, 1979; Vaijayanthimala *et al*., 2000.
Antihelmenthic	Duke and Ayens, 1985; Latha *et al*., 2000.
Antiinflammatory and antipyretic	Backhouse *et al*., 2001.
Antimicrobial	Dev, 2008; Duke, 2009; Khatune *et al*., 2004; Newton *et al*., 2002; Satyavati *et al*., 1987; Yin *et al*, 2004.
Antioxidant	Guo *et al*., 2005; Haraguchi *et al*., 2002;Jiangning *et al*., 2005.
Antitumor	Bapat *et al*., 2005; Haraguchi *et al*., 2002; Latha, 1996; Latha *et al*., 2000;
Cytotoxicity	Kubo et al., 1989; Latha and Panikkar, 1998; Duke, 2009.
Estrogen activity	Dong et al., 2007; Zhang *et al.*, 2005; Zhao *et al*., 2007.
Oestrogenic activities	Lim *et al*., 2009;; Tsai *et al*., 2007; Wang *et al*., 2001b; Xin *et al*., 2009.

1.1.3. Patents on *Psoralea corylifolia* L.

There are several international patents in US, Canada and Europe that deal with the preparation and applications of bakuchiol. An indian patent [Ranganathan,

2006] claims a chemical process to obtain pure bakuchiol. International patents WO/2006/122160 [Jia and Hong, 2006] and WO/2007/147330 [Guo *et al*., 2007] deal with isolation and purification of bakuchiol and its application for treating psychopathy or the neuropathy. An US patent claims a pharmaceutical composition containing bakuchiol to treat breast cancer in women [Lin *et al*., 2005]. There are US and European patents claiming usefulness of bakuchiol composition for treating osteoporosis in women [Lin *et al*., 2006]. An international patented sunscreen composition consisting of bakuchiol claims skin protection from sun-induced damages [Chaudhuri, 2008]. A recent Korean patent claims high estrogenic activity for the extract of *Psoralea corylifolia* [Kim *et al*., 2009].

1.2. Supercritical fluid extraction of bioactive components

There are several methods for extraction of bioactives from natural plant materials like solvent (polar and nonpolar) extraction and distillation. These processes are limited by numerous disadvantages: decomposition of thermally labile bioactives because of high temperature and long duration of heat treatment, non-selective extraction of bioactives. Consequently, the resulting extracts are not as pure as synthetic chemical compounds. The solvents used include hydrocarbons such as hexane, acetone, and methyl chloride which leave unwanted residues in the food products, which are prohibited in some instances by regulatory bodies for use in food. The solvents used for extraction must meet the legal requirements that vary from country to country. Some of the requirements are: high degree of purity, chemical stability, inert (no reaction with food constituent), low boiling point, and no toxic effects. Criteria for these regulations are set by national and international bodies such as the US Food and Drug Administration (FDA), European Economic Commission (EEC) Codex committee, the Canadian Food Inspection Agency (CFIA) and FAO/WHO Codex Alimentarius Commission. Most countries have regulations stating which extraction solvents are generally regarded as safe (GRAS). The possibility of solvent residues remaining in the final product has been a growing concern to consumers, thus warranting stringent environmental regulations. The capability of some supercritical fluids for replacing toxic industrial solvents [DeSimone, 2002], the ability to make new materials at mild conditions and the possibility of turning solvent characteristics for highly specific reactions or separations are some main considerations that underlie current interest in industrial and scientific research in

supercritical fluids [Eckert *et al*., 1996]. The demand for ultra pure and high added-value products is redirecting the focus of food and pharmaceutical industries into seeking the development of new and clean technologies for their products. Many research reviews and books have appeared in recent years which indicate increased research activity in natural product extraction by super critical fluid extraction (SCFE) technology [Martinez, 2008; Shi and King, 2007; Reverchon and DeMaco, 2006; Mukhopadhyay, 2000]. In view of the many positive aspects of SCFE technology, SCFE process is an excellent choice for extracting important bioactives from *Psoralea corylifolia*.

1.3. Molecular distillation

Molecular distillation, also known as Short Path Evaporation, can be considered as a special version of evaporative distillation in which liquid is evaporated without boiling but, that evaporating molecules reach the condenser surface without obstruction. Theoretically, the distance between evaporating and condensing surfaces is kept less than the mean free path of the evaporating molecules so that the molecules reach the condensing surface without colliding with the residual gas molecules [Gruenwedel and Whitaker, 1987; Sattler and Feindt, 1995]. The development of molecular distillation as a separation method started in early thirties and review of the research papers appeared as early as 1940-41. Development in the field of molecular distillation theory and design of equipment of molecular distillation has been well covered by several books [Burrows, 1960; Watt, 1963; Perry and Weissberger, 1965; Hollo *et al.*, 1971]. It is one of the best methods for the separation of heat-sensitive, high molecular weight and low-volatility or high boiling materials by distillation, by which thermal decomposition can be avoided or reduced to a minimum [Sattler and Feindt, 1995]. Typical applications that employ molecular distillation include: pharmaceutical; biomaterial concentration; polymer devolatilization; distillations of esters, fatty acids, mono, di, & triglycerides; residue removal / decolorization; vitamin, nutraceutical, essential oil isolation; wax fractionation; oil deodorization; insecticide removal; water removal at ppm level; food and flavor purification; residual solvent recovery. The molecular distillation technique can be effectively applied to separate bioactives and enrich the extract obtained by SCFE method.

1.4. Enzymatic synthesis of glycosides

Production of fine chemicals results in generation of considerable waste as chemical synthesis generally includes number of steps. Typically, the chemical processing results in one kg of end product leading to generation of 15 kg of wastes. Most of the wastes are solvents and by-products. Biocatalysis has become a preferred method for enzymatic reactions to produce biochemicals. Manufacturing processes in liquid and supercritical fluids (SCF) tend to have advantages in terms of energy reduction, ease of product recovery, lower cost of downstream processing and reduction in side reactions. One-step enzymatic glycosylation is useful for the preparation of glycosides rather than chemical glycosylation which requires large number of protection-deprotection steps.

Glycosylation is the process that links sugar molecules to produce glycans, either free or attached to proteins and lipids. Glycosylation improves the pharmacological property by increasing the water solubility of principal compound. Bakuchiol, the key component of the *P. corylifolia* seeds exhibits poor water solubility, stability and absorbability. Bakuchiol glycoside formed by the process of glycosylation is very much desired to widen the usage of bakuchiol in pharmaceuticals applications better.

1.5. Scope of the thesis

The main objective of the present investigation is to extract bakuchiol, an important bioactive component in *Psoralea corylifolia*. In view of the importance of *P. corylifolia* in nutraceuticals, the principal bioactive components from the seed were extracted by means of advanced separation technology, supercritical carbon dioxide ($SCCO_2$) extraction. As the bioactives of the plant are thermally labile, supercritical CO_2 extraction is highly beneficial process to obtain extracts from *P. corylifolia* seeds. $SCCO_2$ extraction was carried out at various pressures. A theoretical background of supercritical fluids and experimental study of extraction by $SCCO_2$ and modeling of extraction was exhaustively dealt with.

In order to understand extraction process in detail, it is essential to know critical properties of key components of *P. corylifolia* which help in optimizing process conditions and prediction of solubility of key components in supercritical CO_2. Hence, prediction of key components of *P. corylifolia* seeds was determined

based on various group contributions methods such as Lyderson, Ambrose, Joback and Kliencewicz-Reid. Vapor pressure – temperature relationship of bakuchiol and psoralen is also predicted based on well known Riedel method. Progress of the extraction of *P. corylifolia* seeds with supercritical CO_2 is regressed with various extraction models available in literature: Diffusion model, Exponential model, Langmuir and Logistic model. The modeling of extraction helps in the design of commercial supercritical extraction units. Experimental solubilities of key components were predicted based on theoretical solubilities following thermodynamic equation of state models such as Peng-Robinson (PR) and Soave-Redlich-Kwong (SRK). The extract obtained by $SCCO_2$ was also characterized for its bioactive components by HPLC and NMR techniques.

The extracts so obtained by $SCCO_2$ method was further concentrated to contain enhanced amount of the key component of the *P. corylifolia*, bakuchiol, by the technique known as molecular distillation. The effect of process conditions (temperature, vacuum and wiper speed) of molecular distillation was studied based on Box-Behnken design of experiment and response surface methodology (RSM). The resulting second order polynomial model was solved for maximizing bakuchiol content in the extract.

Bakuchiol is an organic compound less soluble in water. It is desirable that bakuchiol extract is made water-soluble for easy application in pharmaceutical formulations. To enhance the solubility of bakuchiol in water and bio-availability, the preferred method is the glycosylation of phenolic –OH group of bakuchiol. D-glucose, D-galactose, D-mannose, D-fructose, D-ribose, D-arabinose, D-sorbitol and D-mannitol are the different carbohydrates selected for the synthesis of bakuchiol glycosides. Various glycosides of bakuchiol were prepared by enzymatic glycosylation based on conventional reflux method and supercritical CO_2 media. Antioxidant and enzyme inhibitory activities of bakuchiol and its glycosides were also determined.

CHAPTER 2

Supercritical fluid Extraction and Characterization of the extract

2.0. Introduction

Organic solvents such as acetone, ethyl acetate, methanol, dichloromethane, and so forth, have been successfully used to isolate bioactive components from hops, spices, oil seeds, and other plant products. Conventional extraction methods using organic solvents are not being preferred because of carcinogenic nature of organic solvents used in the extraction process. Under the Federal Resource Conservation & Recovery Act of USA, industries that use organic solvents must comply strict regulations concerning on-site storage, recycling/disposal and off-site waste transport (www.epa.gov/dfe/garment/frapc/petrfact.htm). National Science Foundation (NSF), USA in its efforts to eliminate/reduce use of chemicals that pose health and environment hazards, award several millions of dollars for funding fundamental research in green chemistry throughout the world (www.epa.gov/opptintr /p2home/ p2policy/ act1990.htm). Among recent advances in the usage of non-conventional extraction methods, supercritical fluid extraction (SCFE) technology is in the forefront. The SCFE technology using CO_2 as supercritical fluid (SCF) has made commercial success in applications such as decaffeinating coffee, tea, and other bioactive (essential oils from spices) components and has the potential to replace conventional separation techniques such as distillation, organic solvent extraction to extract bioactive components from plant sources [Rozzi and Singh, 2002]. As the bakuchiol is thermally labile and forms cyclobakuchiol under heat and acidic conditions [Backhouse *et al*., 2001], supercritical CO_2 extraction is highly beneficial process to obtain extracts from *Psoralea corylifolia* seeds.

2.1. Supercritical fluids

Even though solvating power of super critical fluids has been demonstrated (Hannay and Hogarth, 1879) more than a century back, supercritical fluid techniques are widely used in analytical as well as on an industrial scale since the commercial success of decaffeination of coffee [Zosel, 1978]. The supercritical state is achieved when the temperature and the pressure of a substance is raised over its critical point. Figure 2.1 represents such a phenomenon, which is a phase diagram of a typical substance such as carbon dioxide and water. If we move upward from the gas-liquid coexistence curve, which is a plot of vapor pressure Vs temperature, both temperature and pressure increases.

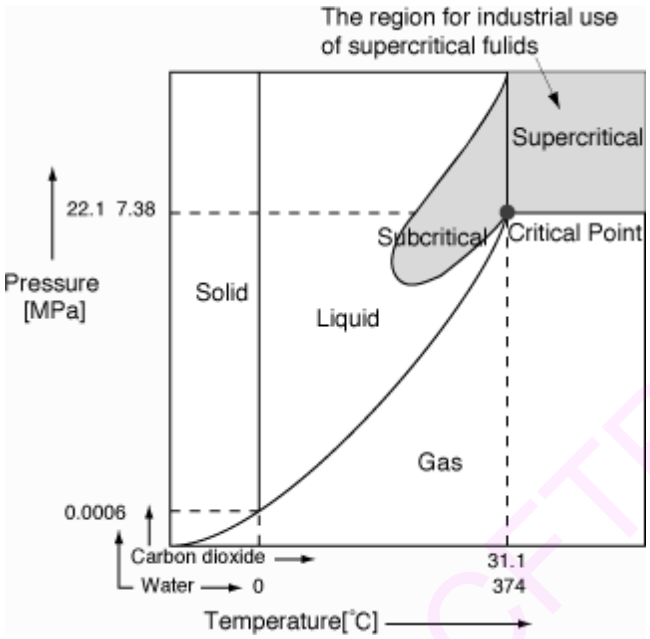


Fig. 2.1. Phase diagram of a typical substance

The liquid become less dense because of thermal expansion and gas becomes denser as the pressure rises. Eventually, the densities of the two phases become identical at the critical point with temperature T_c and pressure P_c. For CO_2, T_c is 31.1°C and P_c is 7.38 MPa while for water, T_c is 374°C and P_c is 22.1 MPa. In the supercritical state the distinction between the liquid and the gas phase disappears and liquid can no longer be liquefied by raising the pressure nor can gas be formed on increasing the temperature. This nature of supercritical fluid arises from the fact that the gaseous and liquid phases merge together and become indistinguishable at the critical point or value. Therefore, the properties of supercritical fluid are intermediate between those of a gas and a liquid (Table 2.1). The densities and viscosities of SCFs are less than liquids and the diffusivities of SCFs are greater than that of liquid.

Table 2.1. Comparison of typical values of physical properties of fluids

Physical Properties	Gases	Supercritical Fluids	Liquids
Pressure (MPa)	0.1/298	P_c/T_c	0.1/298
Density, (kg/m^3)	0.6-2.0	200-800	600-1600
Diffusivity, (cm^2/s)	0.1-0.4	0.7×10^{-3}	$2x10^{-6} – 2x10^{-5}$
Viscosity, (g/cm s)	$(1-3) \times 10^{-4}$	$(1-3) \times 10^{-4}$	$(0.2-3) \times 10^{-2}$

Source: Brunner, 1994.

From the discussion above, it is apparent that the physicochemical properties of a given liquid, such as density, diffusivity, dielectric constant and viscosity can be easily controlled by changing the pressure or the temperature without ever crossing phase boundaries. The dissolving power of a supercritical fluid depends on its density, which unlike that of liquid solvents is highly adjustable by changing pressure or temperature. In the vicinity of the critical point, a small isothermal rise in pressure increases the fluid density remarkably while the effect diminishes with increasing distance from the critical values. Thus, the solvent strength of a fluid can be controlled more easily than that of a liquid solvent. On the other hand, a supercritical fluid has a higher diffusion coefficient, lower viscosity and surface tension than a liquid solvent, which lead to a more favorable mass transfer. Interest in supercritical fluids and its related aspects has made phenomenal growth as seen from the volume of literature available [Knox, 2005]. The close relationship between the fluid density and its dissolving power and its favorable mass transfer properties makes supercritical fluid a useful processing medium for extraction and separation.

2.1.1. Carbon dioxide as an extraction solvent

Even though there are a large number of compounds like ethane, ethene and propane that can be used as a fluid in supercritical processes [Klesper, 1980;

Reglero *et al*., 2005], many of them have a disadvantage of being flammable and being less environmental friendly. The most widely used fluid used as supercritical solvent is carbon dioxide. Probably the most important advantage is that it is a Generally Regarded As Safe (GRAS) solvent that leaves no traces in the product. After extraction, the carbon dioxide is recycled and any trace carbon dioxide in the product dissipates to the atmosphere within few hours. Another advantage of supercritical fluid extraction is the capability of fractionating products to create co-products. Organic solvent extraction requires a distillation step, (in which top notes are lost and distillation notes are created) which, in several cases alters the taste, aroma and chemical composition of the product. Also, trace quantities of residual organic solvent are usually present in the product.

CO_2 is obtained as a by-product of fermentation, combustion and ammonia synthesis. Table 2.2 compares some physical properties of CO_2 Vs other typical solvents [Perry and Green, 1999]. Figure 2.2 shows self-diffusivity of CO_2 which is several orders higher than the typical diffusivities [Mchugh and Krukonis, 1994]. The critical density of CO_2 being one of the highest among the group listed in Table makes it favorable solvent for extraction. The density changes sharply with slight changes in temperature or pressure (Fig 2.3). The behaviour of static dielectric constant with pressure shows similar trends. Both density and dielectric constant rise sharply between 70 and 200 bar and reach values similar to that of liquids. This provides an explanation as to why $SCCO_2$ exhibits high solvent power above a certain pressure, depending on what needs to be dissolved, and thus can be used as a good solvent in place of conventional organic solvents.

The above features of CO_2 make it suitable for extracting, for example, thermally labile and non-polar bioactive compounds, but, because of its non-polar nature, it cannot be used for dissolving polar molecules. Its polar character as a solvent is intermediate between truly non-polar solvent and weakly polar solvents. Because the molecules of carbon dioxide are non-polar it is often classified as non-polar solvent, but it has some limited affinity with polar solutes because of large molecular quadruple moment.

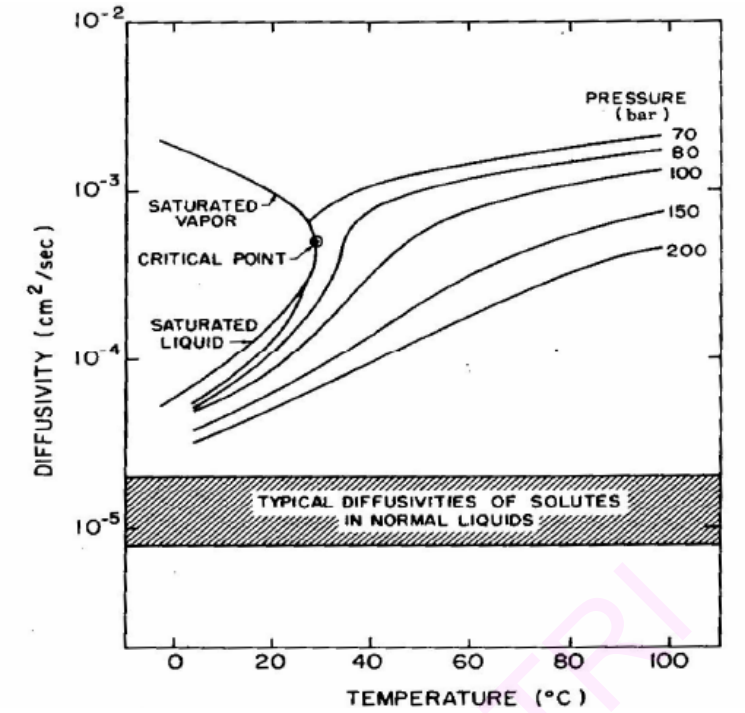


Fig. 2.2. Diffusivity behaviour of CO_2 [Mchugh and Krukonis, 1994].

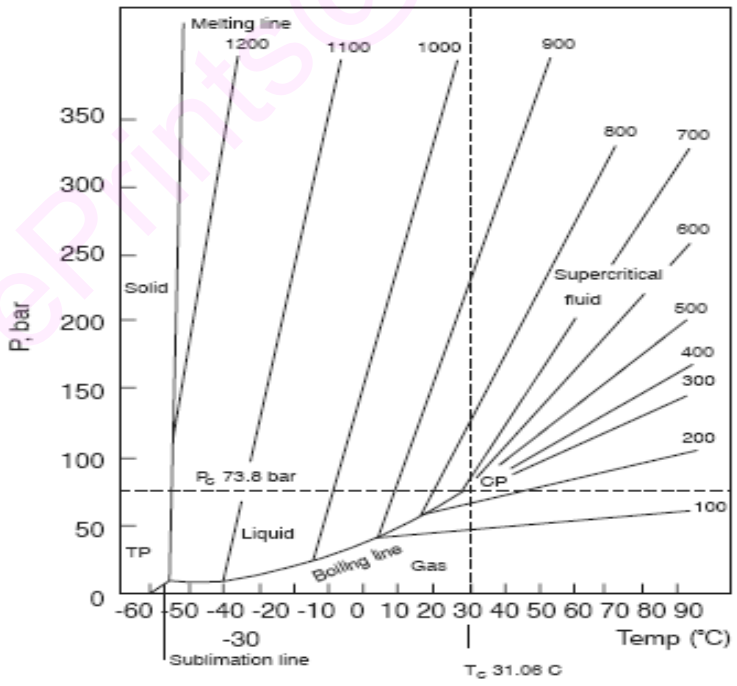


Fig. 2.3. P–T diagram of CO_2 at densities from 100 to 1200 g/L.
(Mchugh and Krukonis, 1994)

Table 2.2. Physical properties of some common solvents in SCF state

Solvent	Critical Temperature (K)	Critical Pressure (MPa)	Critical Density (g/cc)
Acetone	508.1	4.70	0.280
Water	647.3	22.12	0.320
Methanol	512.6	8.09	0.270
CO_2	304.15	7.38	0.468
Tolune	591.75	4.11	0.290
Nitrous oxide	309.65	7.24	0.457

Source: Reid *et al.,* 1989; Perry and Green, 1999.

Supercritical CO_2 tends to be selective towards lower molecular weight compounds (<250) or weakly polar groups such as lipids, cholesterol, aldehydes, ethers, esters and ketones, while high molecular weight (>400) or polar groups such as hydroxyl, carboxyl, and sugars, polysaccharides, amino acids, proteins, phosphatides, glycosides, inorganic salts, are relatively insoluble in dense carbon dioxide [Raventos *et al*., 2002].

The solubility of polar compounds and the selectivity of the process can be increased by adding small quantities of other solvents, such as ethanol in the fluid. On one hand, it decreases the processing times, increases yields and makes it possible to use milder processing conditions, but it also complicates system thermodynamics and increases capital costs [King *et al*., 1993] stated that at high CO_2 densities, its solvent properties were similar to organic solvents like chloroform and acetone, and if intermediate compression were applied, it behaved like a non-polar hydrocarbon such as *n*-pentane or diethyl ether. Advantages of adopting $SCCO_2$ for extraction over conventional solvent extraction are detailed in Table 2.3.

Table 2.3. Advantages of $SCCO_2$ extraction Vs conventional solvent extraction

$SCCO_2$ extraction	conventional solvent extraction
The process can be made selective depending on pressure and temperature. Extracts are mostly pure and concentrated.	Organic solvents are not selective. The process contaminates the extracts with unwanted compounds, heavy metal and other residues.
SCFE is a single step process where extraction and separation of solutes is simultaneous.	The process requires secondary processing for purification from pesticide, solvent residue etc.
CO_2 is GRAS certified. It is non-toxic, non-flammable and free of bacterial content.	Organic solvents are toxic and hazardous beyond certain concentration levels.
Process is gentle on active ingredients since extraction is at high pressure and low temperature. The product is odor-free, stable and has a long shelf life.	Processes involve separation, washing and prolonged heating. Damages active ingredients. Carry odor.

Although CO_2 is often used as supercritical extraction solvent in practice for environmental reasons, the more fundamental interest in supercritical fluids arises because they have properties intermediate between those of typical gases and liquids.

2.1.2. Literature survey on SCFE of bioactive components

Importance of nutraceutical products in human health has been well recognized by industries and researchers in the field of supercritical fluid. This fact can be judged from the amount of literature emerging in recent years. Advances in supercritical fluid extraction of nutraceuticals and bioactive components have been well reflected in a recent book [Martinez, 2008]. SCFE of bioactives such as carotenoids, polyunsaturated fatty acids (PUFA), squalene, sterols and tocols have been dealt [Termelli *et al.*, 2008]. Detailed coverage on extraction of natural tocopherols by $SCCO_2$ is presented [Fang *et al*., 2008]. Bioactive from fish oils such as Omega-3 fatty acids, squalene, diacyl glyceryl ethers and vitamin A (Retinol) using

SCFE has been reviewed [Eltringham and Catchpole, 2008]. Bioactives from algae by supercritical CO_2 extraction has been studied [Mendes, 2008].

There are excellent reviews [Eckert *et al*., 1996; Shi and King, 2007] dealing with various aspects of supercritical fluid extraction. SCFE from food, pharmaceutical, nutraceutical and other natural and biological products has received significant attention in recent years. Rozzi and Singh (2002), Mohamed and Mansoori (2002) and Raventos *et al.*, 2002 reviewed applications of supercritical fluids in the food industry. Rizvi (1994), Awasthi and Trivedi (1997), as well as Mukhopadhyay (2000), enumerated extraction techniques for various types of natural materials. King and List (1996) dealt with applications for SCFE for lipids and oils. Chen and Ling (2000), as well as Lang and Wai (2001) and Catchpole *et al.,* (2004) described applications of SCFE technologies for herbal medicine. Apart from these detailed reviews and books, there are several other research publications on supercritical fluids for the extraction of various biological materials [Froning *et al.*, 1990; Peker *et al.*, 1992; Bhaskar *et al.*, 1993; List *et al.*, 1993; Tsuda *et al.*, 1995; Chester *et al.*, 1998; Cheung *et al.*, 1998; Nguyen *et al.*, 1998; Ambrosino *et al.*, 1999; Cheng *et al.*, 1999; Galan *et al.*, 1999; Ibanez *et al.*, 1999; Senorans *et al.*, 2001; Wong *et al.*, 2001; Canela *et al.*, 2002; Prieto *et al.*, 2003]. A review on future directions of the process SFE has been discussed [Decastro and Carmona, 2000)]. List of various bioactive components which have been reportedly extracted with $SCCO_2$ and the process parameters such as temperature, pressure, CO_2 flow etc., have also been tabulated [Shi *et al*., 2007]. Some studies have focused on other applications, especially with botanical materials and thermally liable substances [Goto *et al*., 1993; Walker *et al*., 1999; Rizvi *et al*., 1994; Rozzi and Singh, 2002; Brunner, 1995; Moura *et al*., 2005; McHugh and Krukonis, 1994]. Many excellent review articles of supercritical fluid technology exist. Subjects covered include: a general overview [Chester *et al*., 1998], the food industry [Brunner, 2005; Rozzi and Singh, 2002], herbal and natural products [Lang and Wai, 2001; Sovova, 2005]; Chinese herbal medicine [Chen and Ling, 2000)] pharmaceutical research [Subramaniam *et al*., 1997; Vasukumar and Bansal, 2003], biotechnology [Williams and Clifford, 2000].

Some of the nutraceuticals products made by supercritical CO_2 extraction and its salient features are given below:

1. Extracts of chamomile flowers for anti-inflammatory and anti-spasmodic bioactive compounds (e.g. sesquiterpene, lactone, matricin). [Scalia *et al*., 1999].
2. Extracts of turmeric for bile preparations - no artifacts such as tolylmethylcarbinol created in steam distillation. [Gopalan *et al*., 2000].
3. Valarian as a sedative preparation - valepotriates obtained undecompossed and at high yield (>90%). [Zizovic *et al*., 2007].
4. Wormwood extract as a carminative, cholagogue and stomachic - removal of toxic β-thujone by fractional extraction from thermally unstable pharmacology active components [Ghasemi *et al*., 2007].
5. Hydrogenation reactions in supercritical carbon dioxide that are a factor of 1000 faster than conventional hydrogenation reactions with greater control over *trans* isomer formation [Marentis *et al*., 2001].
6. Saw Palmetto - higher concentration of phytosterols (active ingredients) [Catchpole *et al*., 2002].
7. Ginseng - extraction of pesticides without extracting significant quantities of active ingredients [Wang *et al*., 2001a].
8. Echinacea - more concentrated extract obtained by SCFE than conventional technologies [Catchpole *et al*., 2002].
9. Feverfew - more concentrated extract obtained by SCFE than conventional technologies [Cretnik *et al*., 2005].
10. Chitin (glucosamine)- able to separate astaxanthin co-product from chitosan using SCFE, able to demineralize shells, and other processing steps [Lim *et al*, 2002].
11. St.John's Wort - more concentrated extract obtained by SCFE than conventional technologies. [Catchpole *et al*., 2002]
12. Kava-kava - more concentrated extract obtained by SCFE than conventional technologies. [Khorassani *et al*., 1999].
13. Gingko biloba - SCFE reduces allergenic compounds in extract. [Yang *et al*., 2002].
14. Garlic (allicin) - SCFE extract more concentrated and deodorized plus higher yields when compared to conventional technologies [Del Valle *et al*., 2008].
15. Evening Primrose oil - more concentrated extract obtained by SCFE than conventional technologies [Favati *et al*., 1991].

16. Rosemary extract - SCFE extract more concentrated and deodorized plus higher yields when compared to conventional technologies [Ibanez *et al*., 1999].
17. Grape seed extract - more concentrated extract obtained by SCFE than conventional technologies [Murga *et al*., 2000].
18. Separation of essential oil from spices without contamination [Udaya Sankar and Manohar, 1998a].

2.1.3. Commercialization of SCFE technology

The early studies on SCFE did not just remain as laboratory curiosity, but resulted in the establishment of large commercial enterprises based on SCFE using carbon dioxide in Europe and USA. The first commercial supercritical fluid extraction was performed in Germany in 1978 by Hag A.G [Palmer and Ting, 1995] for the decaffeination of green coffee beans. Two years later, Carlton and United Breweries in Australia developed a process for the extraction of hop flavors using liquid carbon dioxide [Palmer and Ting, 1995]. Since then, several commercial plants handling several materials started. Commercial applications of the SCFE technology remain limited to a few high-value products due to high capital investment, its novelty, and complex operating system. Adoption of the technology is on the rise as a result of advances in processing, equipment, and the realization of producing high-value products with high profitability. Global perspectives for extraction of high value natural bioactives using SCFE technology has been well covered [Udaya Sankar and Manohar, 1998b]. Partial list of commercial plants employing SCFE technology processing food materials is listed in Table 2.4. During the last 2 decades, a large number of industrials plants (approx. 100) of different volume sizes were built for batch extractions of solid materials with SCF. In this time, a total number of about 100 extractor vessels larger than 100 liters have been designed for different industrial plants, distributed mainly in Europe, Japan, USA and in other Asian countries [Brunner, 2005]. Some of these plants (particularly for hops, tea and coffee) have production capacity at 15-30 million kg per year.

In India, there are few commercial plants mostly dealing with spices and other bio-active materials. In the last decade, many commercial SCFE plants with both imported and indigenous technology processing varieties of raw materials

(particularly, spices) have been established in India. Notable among them are: M/s Flavex Aromats India Ltd, Pune; M/s Novo Agritech., Hydrabad; M/s RKS Agrotech Ltd., Bangalore; M/s SAMI Labs, Bangalore; M/s SouthEast Agro Industries Ltd., Mysore.

Table 2.4. Commercial SCFE plants for extraction of various materials.

	Process	Manufacturer
1	Coffee Decaffeination	Kaffee HAG AG, Bremen, Germany
		General Foods, Houston, Texas
		Hermsen, Bremen, Germany
		Jacobs Suchard, Bremen, Germany
		SKW-Trostberg, Poszzillo, Italy
2	Tea Decaffeination	SKW-Trostberg, Munchmuenster, Germany
3	Fatty Acids from spent Barley	Marbert GmbH, Dusseldorf, Germany
4	Nicotine Extraction	Philip Morris, Hopewell, Virginia
		Nippon Tobacco, Japan
5	Rose-Residual Oil	Kerr-McGee, Oklahoma City, Oklahoma
6	Extracted Pyrethrum	Agrofarm, United Kingdom
7	Hops Extraction	Pfizer Hops Extraction, Sydney, Nebraska
		Hopfenextraktion, HVG, Barth, Raiser & Co.
		SKW Trostberg, Munchsmunster, Germany
		Natal Cane By-Products Ltd., South Africa.
		Barth & Co., Wolnzach, Germany
		Hops Extraction Corp. of America, Yakima, Washington
		J.I. Haas, Inc., Yakima, Washington
		Pitt-Des Moines, Inc., Pittsburgh, USA
		Carlton, United Breweries, United Kingdom
		NORAC, Canada
8	Hops Extraction and Spices	SKW-Trostberg, Munchmuenster, Germany
		Pauls & White, Reigat, United Kingdom
		Nan Fang Flour Mill, China

9	Flavors Extraction	Camilli Albert & Louie, Grasse, France
		Soda Flavor Co., Japan
		Guangxia Toothpaste, China
		Flavex, Rehlingen, Germany
10	Flavors/Aromas/	Flavex GmbH, Rehlingen, Germany
	Spices	Raps & Co., Kulmbach, Germany
		Shaanxi Jia De Agriculture Eng. Co., Ltd., China
11	Corn Oil	Mohri Oil Mills, Japan
12	Color Extraction –	Mohri Oil Mills, Japan
	Red Pepper	Fuji Flavor, Japan.
		Natal Cane By-Products Ltd. South Africa.
		Sumitomo Seiko, Japan
		Yasuma (Mitsubishi Kokoki facility), Japan
		Hasegawa Koryo, Japan
		Takasago Foods (Mitsubishi Kokoki facility), Japan

Source: Udaya Sankar and Manohar, 1998b.

2.1.4. Extraction process and modeling

In Fig. 2.4, a general flow sheet of a supercritical fluid extraction is shown. A typical processing line mainly consists of a pressurizing device (compressor/pump), a pressure vessel for extraction, one pressure vessel for separation (i.e., solvent recovery), a throttling valve for depressurizing and a couple of heat exchangers. Separation is carried out at lower pressure than extraction. The type of pressurizing device used to recirculate the fluid by rising pressure back to extraction conditions depends on the state of the solvent fluid coming from the separator. If the pressure in the separator is high enough for the fluid to be liquefied by chilling in a reasonable temperature range, piston pumps may be applied.

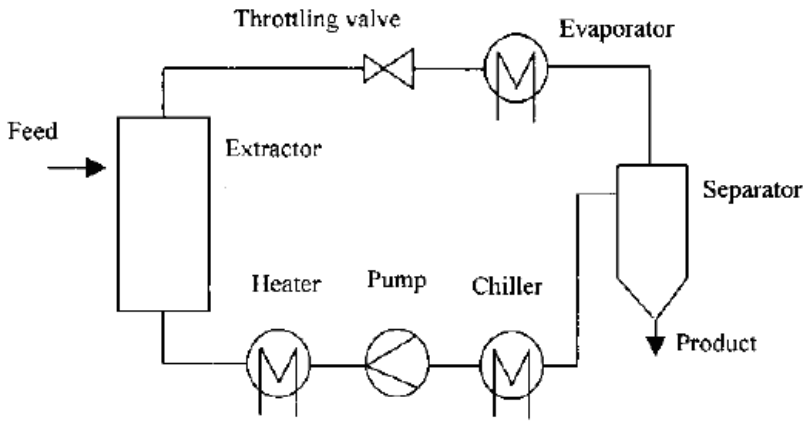


Fig. 2.4. General flow sheet of supercritical fluid extraction

On the other hand, gas compressors working with much lower volumetric efficiencies are needed if the fluid at the pump inlet is in a gaseous state. Pressure must be reduced considerably for precipitating these solutes. Alternative methods of solvent recovery also exist for a complete isobaric solvent cycle. The solute can either be absorbed by an additional liquid, e.g., water, or adsorbed on a fixed bed, e.g., of activated carbon. Therefore, the pump is just needed for maintaining fluid flow and overcoming relatively low-pressure drops along the processing line.

Optimization of process conditions used in SCFE is of great importance to make SCFE technique economically viable because the cost of investment to build a SCFE system is high. Mathematical modeling of experimental data of SCFE has the objective of determining parameters for process design, such as equipment dimensions, solvent flow rate, and particle size. The modeling of extraction of SCFE process makes possible the prediction of SCFE processes on an industrial scale, through the simulation of overall extraction curves.

Effect of extraction pressure and temperature on extract yield and composition of extract have been studied for several plant matters. But there are plenty of other process parameters like particle size, milling conditions fluid velocity etc., influence the extraction process. Different approaches are available in literature to obtain mathematical models that simulate experimental supercritical fluid extraction from solid materials [Tan and Liou, 1989; Udaya Sankar and Manohar, 1994; Esquível *et*

al., 1999; DeFranca and Meireles, 2000; Cocero and Garcia, 2001; Martinez *et al*. 2003; Sovová, 1994 and 2005]. The selection of a suitable model to describe the extraction depends on: 1. Physical meaning of parameters of a particular model; 2. the simplicity or complex nature of the model - particularly empirical ones; 3. the fact that the model was used for a similar system by researchers; 4. the capability of the model to describe the trend of overall extraction curve.

Almost every model in the literature treats the extract as a pure substance, although it may contain several compounds having different chemical natures. This is more so when applied to natural plant materials. In the present work, apart from several conventional models for extraction like diffusion model, exponential model and Langmuir model, a mathematical logistic model (Martinez *et al*., 2003) is proposed to describe the overall SCFE process. The models can be applied to the supercritical CO_2 extraction of *Psoralea corylifolia* seeds considering the entire extract as a single pseudo-component of the extract.

2.1.5. Prediction of critical properties of bioactive components

Critical properties are necessary for adequate design of reactors and separation equipment, processes involving SCFE and in enhancement of oil-recovery techniques [Henderson *et al*., 2004]. Basic pure component parameters required to characterize components or mixtures for calculation of other properties include the melting point, normal boiling point, critical temperature, critical pressure, critical volume, critical compressibility factor, acentric factor, and several other characterization properties. Critical property data are of great practical importance as they are the basis for the estimation of a large variety of thermodynamic properties using the corresponding states principle. Moreover, critical temperature and pressure data provide valuable information for the regression and prediction of vapor pressures at high temperature and are required by equations of state for the description of pure component and mixture behavior. At the critical point, all components show the same striking anomalies like infinite heat capacity and compressibility. The critical temperature and pressure are extremely well defined. Due to the infinite compressibility, experimental critical densities are usually of lower reliability.

Critical properties of pure and simple compounds such as CO_2, water etc., are available in literature. Unfortunately, bioactive components are not only complex in

nature but also not sufficiently stable at or near the critical temperature, and as a result experimental measurements of their critical properties are extremely difficult, if not impossible. It is therefore very important that prediction methods be followed which are capable of not only reasonably accurate predictions, but which are also reliable with a low probability of failure when applied to extrapolation. Besides experimental methods for determining critical properties, there are several correlating methods available. Property estimation methods have been divided in to six groups: (a) graphical approach, (b) equation-of state approach (c) excess-property approach (d) conformal solution (corresponding states principle) approach (e) thermodynamic-potential approach and (f) group-contribution approach [Li and Kiran, 1990].

Group Contribution methods use basic structural information of a chemical molecule like a list of simple functional groups, adds parameters to these functional groups, and calculates thermo-physical and transport properties as a function of the sum of group parameters. Since the first developments of group contribution methods in 1950s [Riedel, 1954; Lydersen, 1955], a large number of methods have been developed for the estimation of critical property data. While various different approaches can be found in literature, the use of the group contribution concept still seems to provide the most reliable and easy to obtain results. A variety of estimation methods for critical property data are available in the open literature. A broad overview of these methods together with a detailed discussion of their reliability has been given [Poling *et al*., 2000]. A recent report [Nannoolal *et al*., 2007] has given the time-line of various group contribution methods for predicting the critical properties in the form of a table.

2.1.6. Solubility of bioactive components

Solubility of a component is an important criterion affecting feasibility of most of the supercritical fluid extraction processes. Solubility affects yield of extraction, cost of processing and also the size and morphology of the product obtained. The solubility of a material is usually expressed in terms of the overall mole fraction of the solute in SCF phase. The ability of SCFs to dissolve many substances arises from highly non-ideal behavior of SCF. The solubility of a component, as predicted by the ideal gas law, decreases asymptotically with increasing pressure because the solubility is simply the ratio of vapor pressure (P^{sat}) to the total pressure (P). Under supercritical

conditions, however, the solubility is enhanced by several orders of magnitude above that predicted the ideal gas law. The solubility enhancement which occurs in the SCF phase is usually expressed in terms of an enhancement factor (E) which is defined as solubility predicted from the vapor pressure of the solute and ideal gas considerations. The values of enhancement factor typically vary between 10^3 and 10^6, although enhancement factors as high as 10^{10} have been reported for some systems [Jessop and Leitner, 1999].

The quantitative determinations of the solubilities of many solutes in various supercritical solvents established that the solubility of solutes in supercritical gases changes significantly with pressure and temperature and the isobars showed maximum and/or a minimum on the temperature-solubility curve [Chrastill, 1982].

2.1.6.1. Summary of equation of state models

Equation of state (EOS) describes the relationship between temperature, pressure, and volume for a given substance or mixture of substances. Many gasses follow the ideal gas law very closely at sufficiently low pressures. The ideal gas law which is a combination of Boyle's Law and Charles' law was originally determined empirically and is simply given as:

$$p V = n R T$$

p = absolute pressure (not gage pressure)

V = volume

n = amount of substance (usually in moles)

R = ideal gas constant

T = absolute temperature

For fluids under supercritical state, real gas laws need to be applied to reflect the true behavior of a gas better than the ideal gas law by putting in terms to describe attractions and repulsions between molecules. Hundreds of EOS's have been proposed—a few before Van der Waals (1887), but mostly later. Van der Waals equation, two parameter Redlich-Kwong equation, Berthelot equation, Dieterici equation, 3-parameter Clausius equation, Peng and Robinson equation, Wohl equation, Beattie-Bridgeman and Benedict-Webb-Rubin are some the equations of state that represent real nature of gases. There are several review articles on real gases which can be referred for more information [Shah and Thodos, 1965; Ross and

Young, 1993]. There are also several books that deal with these EOS in a detailed way [Kondepudi *et al*., 1998; Hsieh, 1993; Stanley, 1985; Perry and Green, 1999].

2.1.6.2. Literature survey on solubility studies in SCF

A good compilation of experimental solubility data of various components from literature is made available in a recent book [Gupta and Shim, 2007]. The data include wide range of compounds that include inorganic, organic and bio-active components. A separate chapter has been dealt with respect to solubility of food components in super critical processes [Shi and Zhou, 2007]. The calculation of solubility of an organic component in SCF has included both correlative and predictive approaches.

One of the popular empirical models to describe the solubility in supercritical fluid is Chrastil equation [Chrastil, 1982]. The model which is based on the formation of a solvate complex establishes a direct relationship between ln(solubility) and ln(density). The model has been found to be valid for number of compounds such as fatty acids, triglycerides [Üstündag and Temelli, 2004].

These models are summarized in empirical correlations as the Chrastil relation [Chrastil, 1982], cubic equations of state (EOS) such as Peng-Robinson [Peng and Robinson, 1976] and Redlich-Kwong [Redlich and Kwong, 1949]. Soave, 1972 made modification to Redlich-Kwong equation of state (RK-EOS). Modified Peng-Robinson equation of state was applied to determine solubility of monofunctional organic solids in diversified SCFs [Schmitt and Reid, 1983]. Later, Soave, 2000 further modified and presented a simple model based on Redlich-Kwong EOS [Soave, 2000].

Both Peng-Robinson equation of state (PR-EOS) and (RK-EOS) predict the equilibrium state well in the compressed gas region. Although the equations of state mentioned above have good prediction performance, they require a lot of critical physical properties of solutes, which are not easily available and only a few are available in the published literature. Hence, empirical expressions are still widely used by some researchers. For example, empirical equations based on power law and Maxwell thermodynamic relation has been proposed [Saeki, 1995]. It has good agreement with some fluids such as neon, hydrogen, deuterium, and carbon monoxide in the supercritical state. Equations of state have a central role in supercritical fluids because they not only predict the solubility, but also give the qualitative phase behavior. Hence, more complicated theories and more multi-parameter empirical methods

continue to be developed to formulate the state more accurately. Although, presently model accuracy is limited by its character, they can be used to build most of the known binary phase diagrams, which are the fundamentals of supercritical fluid extraction. It must be born in mind that selecting a suitable EOS is a difficult task and no single model will work for all situations [Johnston *et al*., 1989]. However, it is advisable to select the equation of state with least number of adjustable parameters for application to biomolecules [Hartono *et al*., 2001].

The objectives of the present study in this chapter are: a) Estimation of the critical temperature, pressure and volume of key components of chiba seed based on various group contribution methods such as Lyderson [Lyderson, 1955], Ambrose [Ambrose, 1978], Joback [Joback, 1984] and Kliencewicz-Reid [Klincewicz and Reid, 1984]. b) Estimation of vapor pressure of the key components as a function temperature by two different prediction methods. c) $SCCO_2$ extraction studies at different pressures and modeling of extraction kinetics by available models in the literature. d) Prediction of solubility of key components (bakuchiol and psoralen based on PR and SRK equation of state models. e) Characterization of the extract for bioactive components.

2.2. Materials and Methods

2.2.1. Materials

Chiba seed powder is a kind gift supplied from M/s Kumar Organic Production, Bangalore, India. Carbon dioxide (food grade) is obtained from approved local dealer (M/s Kiran Corporation, Mysore, India). Hexane (LR grade), Acetonitrile (HPLC grade) are obtained from Merck (India) Ltd., Mumbai, India and Toluene (Analytical Grade) from M/s Qualigens Mumbai, India.

2.2.2. Estimation of moisture content

The moisture content of the seed powder is determined by toluene distillation method. 5g of seed powder is taken in a round-bottomed flask and about 200ml of toluene were added (along with boiling chips to avoid bumping), and placed in a heating mantle with a regulator. The flask is connected to the condenser through a calibrated water-trap. The seed powder is refluxed for 2-3 hours. The amount of water collected was noted, and the percentage of the moisture in the seed is then calculated. All values reported are in duplicates.

2.2.3. Hexane extraction

The seed powder was extracted in hexane by using Soxtec Extraction system (model: Soxtec System HT2 1045 Extraction Unit**,** Foss Tecator, Sweden). About 5g of seed powder was accurately weighed and initially boiled in hexane for a period of 30min. and later rinsed for two hours. The extract was concentrated to dryness by evaporation using Buchi evaporator (model: RT111) in vacuum to find out the oil percentage in hexane extract. All values reported are in duplicates.

2.2.4. Extraction by supercritical CO_2

The powdered seed samples are extracted on a pilot scale in high pressure system (M/s NOVA Swiss Werke AG, Switzerland) designed for working pressures of up to 1000 bar and temperature up to 100°C. Schematic diagram of the high pressure system with all accessories is shown in Figure 2.5. CO_2 gas after being compressed to the required pressure (which is above critical pressure) is made to contact the powdered seeds in the extractor, extracts the soluble bioactives such as bakuchiol and triglycerides from the seeds. After this, the CO_2 is depressurized resulting in the extracted material deposited in the separator vessel. The CO_2 devoid

of the soluble components is again compressed to the operating pressure and recirculated in the system. The pressure, temperature and frequency of the gas flowing through the flow meter are recorded. From the pre-calibrated chart (provided by M/s NOVA Swiss), the amount of carbon dioxide flowing in the system is computed. The extract is periodically removed from the separator and weighed (±0.1g). The extract samples are also analyzed by HPLC. For modeling of extraction kinetics, extraction is conducted at 3 different pressures 220, 260 and 300 bar and temperature at 40°C. For studies on identification of individual compounds present in the extract, the $SCCO_2$ extraction is carried out at 280 bar and 50°C. The extract is taken for further analysis by LC-MS.

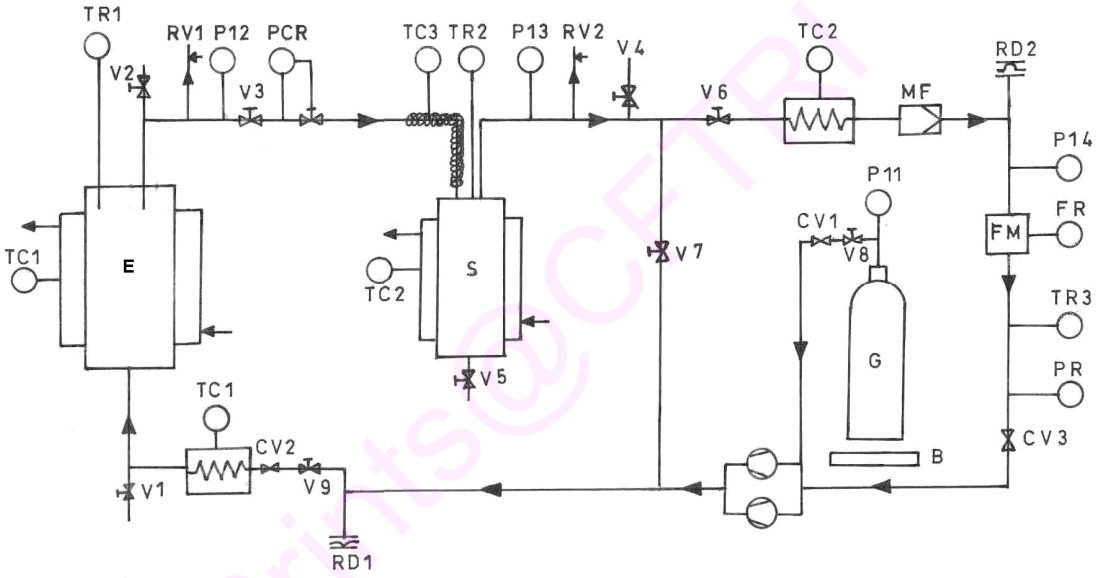


Fig. 2.5. Schematic diagram of the high pressure extraction system

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

2.2.5. HPLC analysis

The HPLC system consists of delivery pumps (Shimadzu LC-10A, Japan), a UV detector and a manual injector valve with a 0.02ml sample loop. Chromatographic separation is carried out at room temperature using a Phenomenex C18 analytical column (250mm x 4.60mm, 5µm). The mobile phase used for the analysis of the extract is an isocratic elution with Acetonitrile-water (70:30) at a flow rate of 1.0

ml/min. The concentration of the sample is 1 mg/ml and injection volume is 20µl. The effluent is detected at 261nm.The results are obtained within 30 minutes.

Purification of bakuchiol and psoralen: About 1 gm of SCF extract is taken and mixed with 4% KOH solution in the ratio of 1:30 and vortexed to provide an emulsion. The mixture is washed with hexane and the organic layer, the emulsified layer and the hexane insoluble layer are separated carefully and the insoluble white precipitate is separated and marked as fraction A. The alkaline aqueous solution is regenerated using carbon dioxide that is separated two phases. The organic layer is extracted with diethyl ether to obtain fraction B. Fraction A was subjected to LC-MS analysis, NMR analysis and proved to psoralen by mass, melting point and NMR spectra. Fraction B is subjected to LC-MS analysis, NMR analysis and proved to be bakuchiol by mass, melting point and NMR spectra.

2.2.6. LC-MS Analysis

The sample solutions are analyzed by Liquid Chromatography - Mass Spectrometry (Waters 2996, photodiode array detector, USA) technique. The mass spectrometry detector is equipped with an ESI (electrospray ionization) source. The ionization mode is negative. Liquid chromatography conditions were same as indicated above. The mass spectrometry conditions are as follows: Capillary, -3.00; Cone, 100; Source temperature, 120°C; Dissolvation temperature, 300°C; Cone gas, 50L/hr; Dissolvation, 500L/hr; scan range, m/z 100-800 with a scan speed of 1000amu/sec. A peak threshold of 1% identity is applied to the mass spectra.

2.2.7. Nuclear Magnetic Resonance (NMR) Spectrum

The purified samples are analyzed by ^{1}H and ^{13}C NMR Spectra of Bruker-500MHz spectrometer. The NMR spectral scans are carried out in deuterated chloroform ($CDCl_3$) solution.

2.2.8. Antioxidant activity measurement

Antioxidant activity of bakuchiol is determined by DPPH (2,2 diphenyl-1-picryl hydrazyl) radical scavenging method [Moon and Tearo, 1998]. The reaction mixture contains 0.1 mL of test sample (5-10mM) and 1.0 mL of DPPH (0.36mM) with the final volume adjusted to 2.0 mL of 0.1M Tris HCl buffer (pH 7.4). The reaction mixture is incubated at room temperature for 20 minutes in the dark and the antioxidant activity is

determined by monitoring the decrease in absorbance at 517 nm on an UV-Visible spectrophotometer (Shimadzu, UV 1601). Butylated hydroxy anisole (BHA- 5.6mM) is used as the positive control. IC_{50} value for the antioxidant activity is expressed as the concentration of the bakuchiol corresponding to 50% decrease in absorbance value of DPPH from a plot of decrease in absorbance versus concentration of the glycoside. Error in activity measurements is ± 5%.

2.2.9. Angiotensin converting enzyme (ACE) inhibition assay

ACE inhibition assay for the bakuchiol is performed on to ACE isolated from pig lung by the Cushman and Cheung method [Cushman and Cheung, 1971]. Aliquots of glycoside solutions in the concentration range 0.2 to 1.8 mM (0.1 mL to 0.8 mL of 2.0 mM stock solution) are taken and to this 0.1 mL of ACE solution (0.1% in 0.1 M phosphate buffer, pH 8.3 containing 300 mM NaCl) along with 0.1 mL of 2.5 mM hippuryl-L-histidyl-L-leucine (HHL) are added and incubated in a water bath for 30 min at 37°C. Blanks are performed without the enzyme. Hippuric acid released is estimated from a calibration plot yielding 0.0105 Abs units/nmol hippuric acid. Percentage inhibition is expressed as the ratio of specific activity of ACE in presence of the inhibitor to that in its absence, the latter being considered as 100%. IC_{50} value is expressed as the concentration of the inhibitor required for 50% reduction in ACE specific activity. Error in measurements is ± 5 %.

2.2.10. Mathematical modeling for extraction kinetics

2.2.10.1. Diffusion model

The result of extraction is a function of how fast the compound is dissolved and the equilibrium in the liquid is reached. Several mass transfer steps are involved in the migration of solute from the solid matrix to the bulk of the fluid/solvent and details on diffusion phenomena are well discussed in several standard books [Treybal, 1896]. In order to simplify the data processing when solute diffusivities in food is undertaken, working in such a way that the rate limiting step is the diffusion of the dissolved solute within the solid into the solvent is advised [Gertenbach, 2001]. The latter approach can be justified when negligible external resistance to mass transfer is supposed. The rate of extraction therefore increases with a larger concentration gradient [Landbo and Meyer, 2001; Pifferi and Vaccari, 1983]. Thus, assuming no

change of the effective diffusivity with solute concentration, the rate of diffusion of solute as the limiting step can be described by Fick's second law.

Considering that particles have spherical shape and concentration differences will be only relevant in radial direction, the Fick's law is written as:

$$\delta C/\delta t = D_e \, \delta^2 C/ \delta r^2 \qquad (2.1)$$

where C is the concentration of the solute, t is the extraction time, D_e is the effective diffusion coefficient or diffusivity, r is the radius of diffusion. Effective diffusivity values can be strongly affected by conditions under which the extraction process is carried out.

Some reports have shown that extraction from food materials is generally controlled by internal diffusion [Dibert *et al*., 1989]. Solutions of Fick's second law are therefore used to determine D_e assuming that D_e is constant with the concentration. Y_D is defined as the ratio between extract concentration at time, t and the initial extract concentration of the matrix. The following boundary conditions will be employed:

$Y_D = 0, r \pm R, t \geq 0$; $Y_D = 1, 0 < r < R, t = 0$.

The solution to eq. (1) is given by:

$$Y_D = (6 / \pi^2) \Sigma (1/n^2). \text{Exp} (n^2 \pi^2 D_e t / r^2) \qquad (2.2)$$

It is generally assumed that, for extraction from plant matrix where external resistance is negligible, the first term of the series solution can usually be used with little error [Schwartzberg, 1975]. Consequently, when the logarithm of Y_D is plotted against time, a straight line should be obtained and the diffusivity can be assessed from its slope.

$$\ln Y_D = \ln (6 / \pi^2) - (\pi^2 D_e t / r^2) \qquad (2.3)$$

The above approach has been especially utilized by a large number of previous works to determine diffusivity values from experimental drying data [Palumbo *et al.,* 1977] or from leaching experiments [Schwartzberg and Chao, 1982]. To avoid the effect of initial operational factors, effective diffusivity values should be predicted by the analytical solution of Fick's second law for large values of time. In this way, besides, Fick's number ($t D_e / r^2$) is greater, and the model could be reduced to the first term accurately [Schwartzberg, 1975].

2.2.10.2. Exponential and Langmuir models

The mathematical relationship between the yield and extraction time gives better insight into the kinetics of the process. Two mathematical functions that are simple to describe are: the simple exponential function and Langmuir's gas adsorption isotherm. They have been used to fit the kinetic curves for liquid CO_2 extraction of essential oils from cardamom, clove, cumin, fennel, ginger, parsley, vetiver and sandalwood [Naik *et al*., 1989].

The exponential model is given in the form:

$$Y = Y_h [1 - \exp (-k_E t)] \quad (2.4)$$

where Y is the extraction yield in weight percent, Y_h is the highest yield, and k_E is a constant. Regression between In $[(Y_h-Y)/ Y_h]$ and t results in $-k_E$ as a slope.

Langmuir's adsorption model, which was found to be more suitable for describing the extraction process of essential oils, is given in the form:

$$Y = Y_f t / (k_L + t) \quad (2.5)$$

where Y is the extraction yield, and t is the extraction time, and Y_f and k_L are constants (Y_f is the yield at infinite extraction time). Equation (5) can be rearranged into the form:

$$1/Y = 1/ Y_f + (k_L / Y_f).(1 / t) \quad (2.6)$$

Regression between 1/Y and l/t would result in k_L / Y_f, as a slope and $1/ Y_f$, as the intercept.

2.2.10.3. Logistic model

The mathematical logistic model in this study is based on the model adopted by Martinez et al., 2003. Only a brief procedure of the method is outlined here whereas for detailed description of the model, one may refer Martinez et al., 2003. The extraction bed is considered to consist of: (a) a solid phase, composed of the raw material from where the extract is obtained, and (b) a fluid phase, composed of the supercritical fluid with the extract dissolved in it. The mass balance in both fluid and solid phases may be expressed by equations 1 and 2, respectively:

$$(\delta Y/\delta t) + u. (\delta Y/\delta h) = \delta(D_{aY} \delta Y/\delta h) /\delta h + J(x,y)/ \varepsilon \quad (2.7)$$

$$(\delta X/\delta t) = \delta(D_{aX} \delta X/\delta h) /\delta h + (J(x,y) / (1-\varepsilon)) . (\rho / \rho_s) \quad (2.8)$$

In equations 7 and 8, X and Y are the solute mass ratios in the solid and fluid phases, respectively; u is the interstitial velocity of the solvent; h is the axial coordinate in the extraction bed; D_{aY} is the axial dispersion coefficient; D_{aX} is the diffusion coefficient in the solid phase; ρ is the solvent density; ρ_s is the solid density; ε is the bed porosity; and J(x,y) is the interfacial mass transfer flux. The logistics model assumes that accumulation and dispersion of solubles in the fluid phase is neglected as these have insignificant influence on the extraction phenomena as compared to the effect of convection. The model also implies that when the extraction time approaches infinity, the mass of the extracted material tends to a fixed value asymptotically. Simplification of differential equation has been detailed elsewhere. The final equation, after integration and rearrangement of the terms, for the extraction curve of a single group of components is:

$$m\,(h=H,t) = [m_t / \exp(b\,t_m)] * [\,(1+ \exp(b\,t_m)) / (1 + \exp\,(b\,(t_m - t))) - 1] \qquad (2.9)$$

The advantage of this rearrangement is that the new parameter has a clear physical meaning and it can be determined experimentally if the total extractable mass and the total composition of the extract are known. In this case, the model can be reduced to two adjustable parameters per group of compounds: b and t_m. Otherwise, if these data are not determined experimentally, the parameter m_t must also be adjusted for each group of compounds. In the present study, the entire extract has been assumed to consist of single component since the extract consists of about 50% Bakuchiol. The model is called as single component logistic model. The parameters of the model were determined by least squares nonlinear regression method. Sum of squared deviation between experimental yield and predicted yield is minimized based on Newton search method. The 'SOLVER' program of the MS-Excel software has been used for determination of the parameters of model.

2.2.11. Prediction of critical properties

2.2.11.1. Critical temperature (T_c): The critical temperature is an important parameter in determining the phase boundaries of any compound and is a required input parameter for most phase equilibrium thermal property or volumetric property calculations using analytic equations of state or the theorem of corresponding states.

Among the various group contribution methods available in the literature, only Ambrose [Ambrose, 1978], Joback [Joback, 1984], Klincewicz-Reid [Klincewicz and Reid, 1984], and Lydersen [Lydersen, 1955] have been included in this study for

estimation of critical temperature. Historically, Lydersen's method, developed in 1955, was found to be the most accurate method available for T_C prediction for hydrocarbons and organics. Very recently, Joback took advantage of the new critical data that has become available since Lydersen's original work in 1955 to modify Lydersen's scheme slightly and develop a method that is as accurate as Ambrose, but again, not as complex. All four of these methods have merit and are therefore considered. Lydersen is still important for comparisons, as it was the standard for over 20 years. Klincewicz-Reid, in their investigation of prediction of critical temperature methods, found that a simple correlation using only the normal boiling point and the molecular weight will give nearly the same result as a complicated group contribution scheme. Equations pertaining to all the four methods are given below:

Ambrose

$$T_C = T_B (1 + \sigma_A + \sum\Delta T_A) / (\sigma_A + \sum\Delta T_A) \quad (2.10)$$

Joback

$$T_C = T_B / [0.584 + 0.965 \sum\Delta T_J - (\sum\Delta T_J)^2] \quad (2.11)$$

Klincewicz-Reid

$$T_C = 45.4 - 0.77 M_w + 1.55 T_B + \sum\Delta T_K \quad (2.12)$$

Lydersen

$$T_C = T_B / [0.567 + \sum\Delta T_L - (\sum\Delta T_L)^2] \quad (2.13)$$

Where T_B = Normal Boiling temperature

σ_A = 1.570 for perfluorocarbons, 1.242 otherwise.

ΔT_A = Group Contributions for temperature in Ambrose equation (2.10)

ΔT_J = Group Contributions for temperature in Joback equation (2.11)

ΔT_K = Group Contributions for temperature in Klincewicz-Reid equation (2.12)

ΔT_L = Group Contributions for temperature in Lydersen equation (2.13)

M_w = molecular weight of the compound.

2.2.11.2. Critical pressure (P_c): Again, only four group contributions methods mentioned earlier have been used for predicting the critical pressure. As with the critical temperature, Lydersen's method has long been the preferred method. Ambrose used the new data measured since Lydersen and improved upon the earlier method. Klincewicz-Reid took a little different approach to their correlation, resulting in a form different than that of Lydersen or Ambrose with simpler group contributions. Joback, as with Ambrose, took advantage of the new data available but kept a form

very similar to Lydersen. As with the critical temperature correlations the Ambrose method is the most accurate but only slightly more so than that of Joback which is much less complex than Ambrose. Klincewicz and Reid or Lydersen methods, while simpler than Ambrose are not as accurate as Joback. Klincewicz-Reid also offers a simple method that does not include a group contribution, but rather requires only the molecular weight and the number of atoms in the molecule. Klincewicz- Reid method will be useful for a quick reference or for the prediction of a compound's critical pressure whose groups are not listed. The equations for predicting critical pressure are listed below:

Ambrose

$$P_C = M_W / [1.01325 (\partial_{PA} + \sum \Delta P_A)^2] \quad (2.14)$$

Joback

$$P_C = [(0.113 + 0.0032 n_A - \sum \Delta P_J)^{-2}]/1.01325 \quad (2.15)$$

Klincewicz-Reid

$$P_C = M_W / [1.01325 (0.348 + 0.0159\ MW + \sum \Delta P_K)^2] \quad (2.16)$$

Lydersen

$$P_C = M_W / (0.34 + \sum \Delta P_L)^2 \quad (2.17)$$

Where ∂_{PA} = 1.0 for perfluocarbons, 0.339 otherwise. n_A = number of atoms in the compound. ΔP_A = Group Contributions for pressure in Ambrose equation (2.14). ΔP_J = Group Contributions for pressure in Joback equation (2.15). ΔP_K = Group Contributions for pressure in Klincewicz-Reid equation (2.16). ΔP_L = Group Contributions for pressure in Lydersen equation (2.17).

2.2.11.3. Critical volume (V_c): The third of the three critical properties is usually the most difficult to measure. So consequently, the database of good experimental critical volume data is smaller. The group contribution methods followed the same development as the critical temperature and pressure. That is, the Lydersen was improved upon by Joback and by the more complex Ambrose method while Klincewicz- Reid somewhat simplified the Ambrose method. Again as with the other critical property methods Klincewicz-Reid presented a simple correlation that does not use group contributions, nor corresponding states methods. This simple method uses only the compound's molecular weight and number of atoms. Again this can be useful if other critical data is not available and the compound structure is not given in any of the group contribution methods.

Ambrose

$$V_C = 40 + \sum \Delta V_A \quad (2.18)$$

Joback

$$V_C = 17.5 + \sum \Delta V_J \quad (2.19)$$

Klincewicz-Reid

$$V_C = 25.2 + 2.8\, M_W + \sum \Delta V_K \quad (2.20)$$

Lydersen

$$V_C = 40 + \sum \Delta V_L \quad (2.21)$$

Where ΔV_A = Group Contributions for volume in Ambrose equation (2.18)

ΔV_J = Group Contributions in Joback equation (2.19)

ΔV_K = Group Contributions for volume in Klincewicz-Reid equation (2.20)

ΔV_L = Group Contributions for volume in Lydersen equation (2.21)

2.2.11.4. Normal boiling temperature (T_B): The normal boiling temperature being very commonly available, it is often used in estimation schemes. Generally measurements of boiling points are made at lower pressures for extremely high boiling compounds or for compounds that decompose (especially true for bio-active components such as bakuchiol) before they boil at atmospheric pressure. Joback used the same approach to develop a method for normal boiling point as he used to develop a method for critical temperature. The resulting method is a function of structure only. The Joback's equation is below:

$$T_B = 198 + \sum \Delta B_J \quad (2.22)$$

Where ΔB_J = Group Contributions for boiling point.

2.2.11.5. Vapor pressure: Vapor pressure is a very useful property that determines the feasibility of separation of the components under separation processes such as distillation. The vapor pressure is the pressure exerted by a pure component at equilibrium at any temperature when both liquid and vapor phases exist and thus extends from a minimum at the triple point temperature to a maximum at the critical temperature, the critical pressure.

Among the various equations available for prediction of vapor pressure, the most important is the Riedel [Riedel, 1954] method which is given below:

$$\log P^s_r = -S_1(T_r) - (S_2 - 7)\, S_3(T_r) \quad (2.23)$$

where correlation functions $S_1(T_r)$, $S_3(T_r)$ and $S_4(T_r)$ are given by:

$$S_1(T_r) = 0.118\ S_4(T_r) - 7\ \log T_r \quad (2.24)$$

$$S_3(T_r) = 0.0364\ S_4\ (T_r) - \log T_r \quad (2.25)$$

$$S_4(T_r) = 36/T_r + 96.7\ \log\ T_r - 35 - T^6_r \quad (2.26)$$

$$S_2 = S_{21} / S_{22} \quad (2.27)$$

where $S_{21} = 0.136\ S_4(T_{rb}) + \log P_c - 5.01$ (2.28)

$$S_{22} = 0.0364\ S_4(T_{rb}) - \log T_{rb} \quad (2.29)$$

where T_r = Reduced temperature (T/ T_C)

T = Temperature of interest

T_{rb} = Reduced temperature at normal boiling temperature (T_B/ T_C)

S_1, S_2, S_3, S_4, S_{21}, S_{22} = constants as defined in equations (2.24) to (2.29).

Another method that is used in the present study is the one developed by Edalat *et al*., 1993. The method is claimed to be accurately predicting vapor pressure of hydrocarbons, non-hydrocarbons and polar fluids using only P_c, T_c and ω, the acentric factor. The equation is described below:

$$\ln P^s_r = [\eta_1\ \tau + \eta_2\ \tau^{1.5} + \eta_3\ \tau^3 + \eta_4\ \tau^6\ ]\ [\ 1- \tau]^{-1} \quad (2.30)$$

where $\eta_1 = -6.1559 - 4.0855\ \omega$ (2.31)

$$\eta_2 = 1.5737 - 1.0540\ \omega - 4.4365 \times 10^{-3}\ \eta_4 \quad (2.32)$$

$$\eta_3 = -0.8747 - 7.8874\ \omega \quad (2.33)$$

$$\eta_4 = [-0.4893 - 0.9912\ \omega - 3.1551\ \omega^2]^{-1} \quad (2.34)$$

$$\tau = 1 - T_r \quad (2.35)$$

$$\omega = (-\log P^s_r)_{Tr=0.7} - 1 \quad (2.36)$$

where P^s_r = reduced vapor pressure (P^s/P_c). η_1 , η_2 , η_3 , η_4 = constants defined in equation in (2.30) to (2.34).

2.2.12. Prediction of solubility

Equations of state are used to describe the thermodynamic states through the relationship of state parameters such as temperature, pressure, and volume. In SCFE processing, EOS describes the behavior of solute being dissolved in supercritical fluids. The solubility of a substance (component 2) in a supercritical fluid (component 1) can be obtained from fluid-solid phase equilibria. The subscripts 1 and 2 in the following derivations denote components CO2 and solute (bakuchiol or psoralen as the case may be). At equilibrium, the fugacities of the components in the solid and gas phases are equal, i.e.:

$$f_2^s(P,T,x_2) = f_2^v(P,T,y_2) \quad (2.37)$$

where f_2^s is the fugacity of solute in the solid phase; f_2^v fugacity of solute in the gas phase; P is the pressure; T is the temperature; x and y are mole fractions.

If the gas component 1 is assumed to be insoluble in solid phase (true in most of the cases), then

$$y_2 \varphi_2^s P = P_2^{sat} \varphi_2^{sat} \exp \int_{P2sat}^{P} ((v_2^s dP/ RT)) \quad (2.38)$$

$$f_2^v = P y_2 \varphi_2^v \quad (2.39)$$

where, φ is the fugacity coefficient; V molar volume; R is the universal gas constant; superscripts s and sat denote solid and saturated conditions respectively.

From above equations, one can obtain:

$$y_2 = (P_2^{sat} / P) E \quad (2.40)$$

$$y_2 = P_2^{sat} \exp (P v_2^s / RT) / (\varphi_2^v P) \quad (2.41)$$

where E is the enhancement factor which is the ratio of the solubility of the solute in super critical fluid to the solubility in an ideal gas. i.e $E = y_2 / y_{ideal}$

Large enhancement factors of the order of 10^5 to 10^7 are frequently observed due to small values of φ_2^v. φ_2^{sat} has a value of approximately one. V_2^s can be easily calculated from the density of component 2. Thus, to calculate E, one requires the value of φ_2^v . From well known equation of state (EOS) available such as Peng-Robinson (PR) and Soave-Redlich-Kwong (SRK) models, φ_2^v can be calculated.

A general form of the Peng-Robinson equation is:

$$P = (RT/(V-b)) – (a / (V^2+2bV – b^2)) \quad (2.42)$$

The above can be written as:

$$Z^3 – (1-B) Z^2 + (A-3B^2-2B) Z - (AB-B^2-B^3) = 0 \quad (2.43)$$

The above equation leads to:

$$\ln \varphi_2^v = b_2 (Z-1)/b – \ln (Z-B) – [(A/2\sqrt{2}B) \{2(a_{22} y_2 + a_{12} y_1 /a) – (b_2/b)\} . \ln \{(Z+2.414 B)/(Z- 2.414 B)\}] \quad (2.44)$$

Soave-Redlich-Kwong equation of state (SRK-EOS) is:

$$P = (RT/(V-b)) – (a / (V^2 + bV)) \quad (2.45)$$

The above can also be written as:

$$Z^3 – Z^2 + (A-B-B^2) Z – AB = 0 \quad (2.46)$$

The above equation leads to:

$$\ln \varphi_2^v = b_2 (Z-1)/b - \ln (Z-B) - [\ (A/B)\ \{2(a_{22}\ y_2 + a_{12}\ y_1 /a\ ) - (b_2/b)\}\ .\ \ln (1 + (B/Z))\ ] \quad (2.47)$$

where $Z = Pv / RT$ (2.48)

$A = a\ P / (R^2\ T^2)$ (2.49)

$B = b\ P / RT$ (2.50)

a = intermolecular attraction parameter

b = covolume parameter

When the equation is extended to mixtures, the following mixing rule is employed:

$$a = \Sigma\ \Sigma\ y_i\ y_j\ a_{ij} \quad (2.51)$$

$$b = \Sigma\ \Sigma\ y_i\ y_j\ b_{ij} \quad (2.52)$$

where a_{ij} and b_{ij} are calculated from combining rules:

$$a_{ij} = (a_{ii}\ a_{jj})^{0.5}\ (1- k_{ij}) \quad (2.53)$$

$$b_{ij} = (b_{ii}\ b_{jj})^{0.5}\ (1- n_{ij}) \quad (2.54)$$

where k_{ij} and n_{ij} are the adjustable binary interaction parameters. The adjustable parameters are determined by regressing phase equilibrium data and assumed to be independent of temperature, pressure and composition. It may be noted that these parameters can take negative values and accordingly, the software program should be allowed to adjust these values suitably. Many authors have used single binary interaction parameter k_{ij} in their works. However, a second adjustable mixture parameter n_{ij} is also used in the present study, since n_{ij} can account for the large size disparity between a heavy non-volatile solute and a light SCF solvent [Mchugh and Krukonis, 1994]. For pure components, 'a' and 'b' parameters are calculated from critical constants T_c, P_c and acentric factor, ω depending on the type of EOS.

For, PR-EOS,

$$a_i = 0.45724\ (\ R^2\ T_c^{\ 2}\ /\ P_c\ )\ \alpha \quad (2.55)$$

$$\alpha = [1+ K\ (\ 1-T_r^{\ 05}\ )\ ]^{\ 2} \quad (2.56)$$

$$K = 0.37464 + 1.54226\ \omega - 0.26992\ \omega^2 \quad (2.57)$$

$$b_i = 0.07780\ R\ T_c / P_c \quad (2.58)$$

For SRK-EOS,

$$a_i = 0.42748\ (\ R^2\ T_c^{\ 2}\ /\ P_c\ )\ \alpha \quad (2.59)$$

$$\alpha = [1+K\ (\ 1-T_r^{\ 05}\ )\ ]^{\ 2} \quad (2.60)$$

$$K = 0.48 + 1.57\ \omega - 0.17\ \omega^2 \quad (2.61)$$

$$b_i = 0.08664 (R T_c / P_c) \quad (2.62)$$

The computer program obtains the optimum k_{ij} and n_{ij} value by simple optimization technique which gives minimum average absolute relative deviation (AARD) between experimental and calculated values of solubility:

$$\text{AARD, \%} = (1/N) \Sigma (y_{exp} - y_{cal}) .100 / y_{exp} \quad (2.63)$$

where N is the number of experiments, y_{cal} and y_{exp} are the calculated and experimental solubilities of solute components respectively.

Experimental solubility is calculated from the slopes of the initial linear portion of extraction curve of total extract versus CO_2 consumed [Liang and Yeh, 1991]. When the extract is assumed as a single pseudo-component, the T_C, P_C, ω, P^{sat} of the extract is considered to be sum of the property of the main components multiplied by mole fraction of main components.

The entire calculation procedure [Basalingappa *et al.*, 2001] described above can be summarized as below

1. The critical values T_c, P_c and acentric factor for each component are estimated by group contribution methods.
2. Pure component parameters of the PR-EOS and SRK-EOS are estimated.
3. k_{ij} and n_{ij} are assumed initial values. Then a and b for mixture are calculated based on mixing rules.
4. The cubic equation of state is solved for Z.
5. φ_2^v is calculated.
6. y_2 is calculated.
7. Steps 2 to 6 repeated for each pressure. AARD is then calculated. If AARD is not within tolerance limit set by the program, the procedure is repeated so that one obtains the optimal k_{ij} and n_{ij} parameters.

A program is written in MS-Excel® incorporating the above steps to calculate the solubility with minimum deviation.

2.3. Results and Discussion

2.3.1. $SCCO_2$ extraction studies and modeling

The *Psoralea corylifolia* seed contained moisture content of 4% w/w. Hexane extraction of the seeds yielded 12.0% kg oil/ kg seed. Bakuchiol content in the hexane extract was 35.2% w/w whereas psoralen and isopsoralen together in the hexane extract was 1.2% w/w. Yield of extract, bakuchiol content in various extract fractions and total bakuchiol content in the final extract during the course of $SCCO_2$ extraction are tabulated (Table 2.5).

Table 2.5. Extract yield and bakuchiol content during SCF extraction

Pressure, bar	Extraction period, s	Bakuchiol in extract, %	Total Bakuchiol in extract, %	Extract yield, % (kg oil/kg seed)
220	21900	60.1	49.0	9.7
	62400	38.6		
	84000	38.7		
260	3600	44.8	41.1	11.5
	6000	55.5		
	12000	61.0		
	15600	56.4		
	19200	50.5		
	22800	46.1		
	30000	34.3		
	51300	40.6		
300	10800	61.0	51.7	11.8
	14400	57.5		
	19800	53.6		
	27000	54.1		
	36000	47.2		
	65100	34.8		

Depending on the pressure of extraction, the yield in $SCCO_2$ extraction process varied from 9.7% to 11.8% kg oil/ kg seed. This $SCCO_2$ yield was lower by

2.1% to 19.1% compared to the yield obtained by hexane extraction method. The yield of extract (by $SCCO_2$ process) increased as pressure of extraction increased. Bakuchiol content in extract fractions at different time periods varied from a low of 34.3% to a high level of 61% during the course of extraction. No particular trend could be noticed with respect to bakuchiol content during the course of time of extraction or pressure of extraction. Overall bakuchiol content in the $SCCO_2$ extract was between 41.1% and 51.7% depending on the pressure of extraction. The bakuchiol content in the extract by $SCCO_2$ process was substantially higher (16.8% to 46.9%) than that bakuchiol content of the extract obtained through hexane extraction method. Since most of the studies available in literature on *P. corylifolia* dealt with identification of compounds and biological activities of the compounds isolated from the seed, no comparative data on extraction yield are available.

Experimental extraction data at various pressures depicting the quantity of extract collected versus CO_2 consumed is in Table 2.6. It was clear from the extraction curves (Fig. 2.6) that the yield of extraction increases as the pressure of extraction increase as solvent density increases thereby aiding the better extraction of solubles. The initial slope of the extraction curve was taken as solubility of the component at the particular pressure. The solubilities so calculated are presented in Table. 2.7.

The extraction followed the linear trend during the initial period and the slope of the curve decreased as the extraction period increased (Fig. 2.6). The solubilities of many natural components in supercritical CO_2 has been compiled [del Valle *et al*., 2005] and it can be inferred that the solubilities vary widely with pressure and temperature of extraction. The solubilities reported in this work ranged from 0.0015 to 0.27 kg solute / kg CO_2.

Table 2.6. Experimental data showing cumulative extract Vs CO_2 consumed

220 bar		260 bar		300 bar	
CO_2 consumed, kg	Cumulative extract, kg	CO_2 consumed, kg	Cumulative extract, kg	CO_2 consumed, kg	Cumulative extract, kg
0.00	0	0	0	0	0
0.97	0.0013	1.43	0.0014	4.49	0.0055
1.94	0.0029	3.10	0.0045	6.14	0.0085
2.91	0.0039	4.89	0.0086	7.32	0.0135
3.54	0.0047	6.47	0.0114	8.90	0.0205
4.79	0.0059	10.45	0.0161	11.40	0.0298
5.67	0.0068	14.26	0.0204	14.85	0.0391
6.55	0.0087	18.56	0.0298	19.65	0.0491
7.28	0.0094	22.78	0.0372	24.75	0.0539
8.06	0.0109	29.79	0.0472	30.45	0.0561
8.97	0.0124	35.16	0.0513	33.88	0.0587
10.52	0.0146	38.76	0.0543		
11.43	0.0165	42.31	0.0573		
12.67	0.0182				
14.00	0.0197				
14.99	0.0210				
15.95	0.0221				
17.14	0.0236				
19.99	0.0263				
22.41	0.0286				
24.56	0.0309				
27.32	0.0320				
29.32	0.0329				
30.90	0.0340				

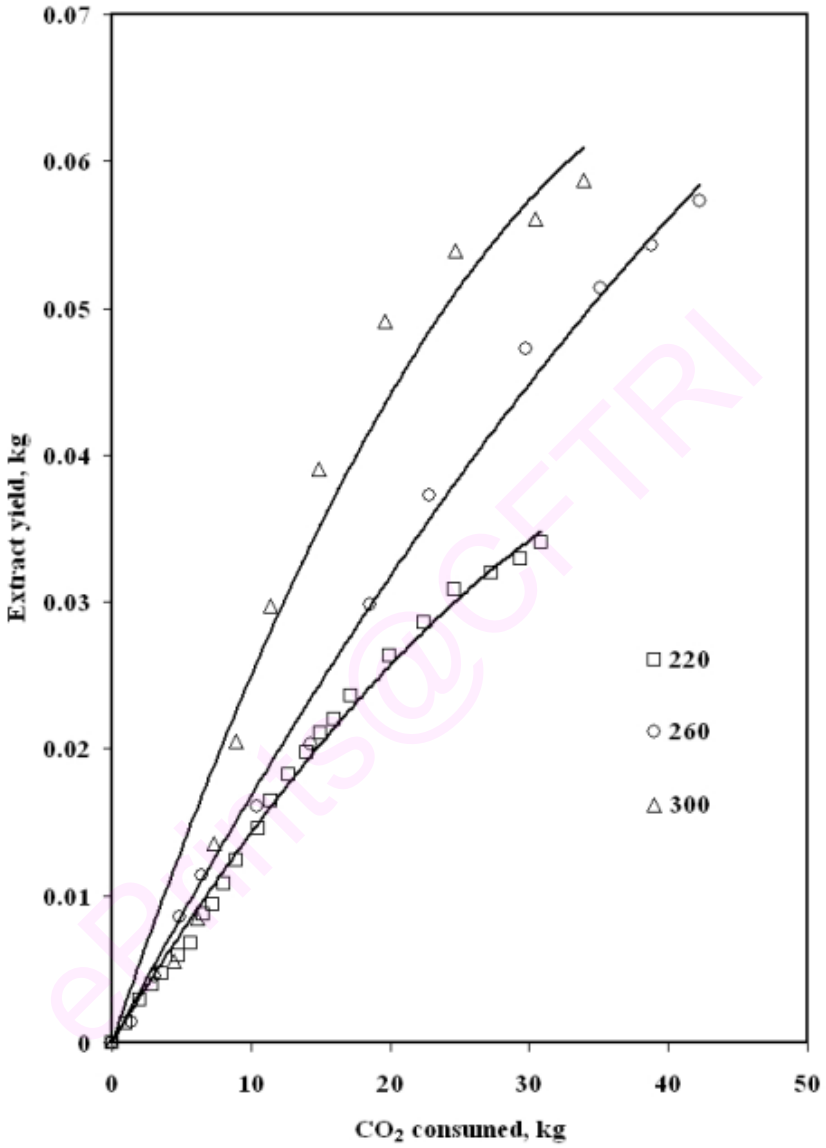


Fig. 2.6. Extraction yield Vs CO_2 consumed at various pressures.

Table 2.7. Parameters of Logistic model for SCF extraction

	Extraction pressure, bar		
Parameters	220	260	300
b	2.05×10^{-05}	9.36×10^{-05}	5.61×10^{-05}
t_m, s	1000	15000	3000
Solubility, kg / kg CO_2	0.0015	0.0018	0.0027
Correlation coefficient	0.95	0.99	0.99
AARD, %	26.1	10.7	15.3
Min. ARD, %	0.02	0.3	1.4
Max. ARD, %	62.7	52.5	51.1

In order to explain the extraction phenomena, experimental extraction data were regressed to diffusion, exponential and Langmuir model and their correlation coefficients for the models with parameters of the models are listed in Table 2.8. Diffusion model (equation 2.3) applied to extraction data for the pressure range is in Figure 2.7. The high correlation coefficient ($R > 0.98$) of the regression indicated that model explained the extraction process well. Effective diffusivity calculated based on the model is in the range 4.4×10^{-11} to 7.2×10^{-11} (Table 2.8).

Effective diffusivity for the extraction of pepper and paprika using CO_2 were reported to be in the range $1.54 \times 10^{-11} - 39 \times 10^{-11}$ m^2/s [Skerget and Knez, 2001]. The diffusion coefficient of menthol, the main constituent of mint oil, in supercritical solvent varied from 0.17×10^{-8} to 1.46×10^{-8} m^2/s [Goto *et al.*, 1993]. Mass diffusivities of basil, marjoram and rosemary essential oils in CO_2 for particles of different sizes at 100 bar and 313 K have been provided [Reverchon *et al*., 1993]. It was found that the effective diffusion coefficient varied from 1.4×10^{-13} to 2.8×10^{-13} m^2/s. These authors indicated that internal resistance in herbaceous matters controls the mass transfer process. Application of diffusion model was studied [Roy *et al.*, 1996] for the extraction of ginger essential oil at different CO_2 flow rates and the estimated effective diffusion coefficient was found to be 2.5×10^{-10} m^2/s.

Table 2.8. Parameters of Diffusion, Exponential and Langmuir model applied to SCF extraction

A. Diffusion model

Pressure, bar	D_e	Correlation coefficient
20	$4.4 \pm 0.1 \times 10^{-11}$	0.98
260	$7.2 \pm 0.1 \times 10^{-11}$	0.99
300	$7.1 \pm 0.1 \times 10^{-11}$	0.99

B. Exponential model

Pressure, bar	k_E	Correlation coefficient
220	$3.3 \pm 0.1 \times 10^{-5}$	0.98
260	$4.9 \pm 0.1 \times 10^{-5}$	0.97
300	$4.8 \pm 0.1 \times 10^{-5}$	0.97

C. Langmuir model

Pressure, bar	k_L	Correlation coefficient
220	$2.1 \pm 0.1 \times 10^{6}$	0.98
260	$4.1 \pm 0.1 \times 10^{6}$	0.96
300	$1.0 \pm 0.1 \times 10^{6}$	0.98

In the present study, the effective diffusion coefficient was found to be in the similar order. The highest D_e was found at 260 bar. The diffusivity in $SCCO_2$, in general, increased with temperature and decreased with pressure. At low pressure, the diffusivity is nearly independent of composition, whereas at higher densities, the composition dependence becomes more significance [Mukhopadhyay, 2000]. The simple exponential model (equation 2.4) fitted the extraction data with correlation coefficient, R >0.97 and the regression plot is shown in Figure 2.8. The Langmuir model (equation 2.6) also fitted the experimental extraction data reasonably well with R >0.96 (Figure 2.9).

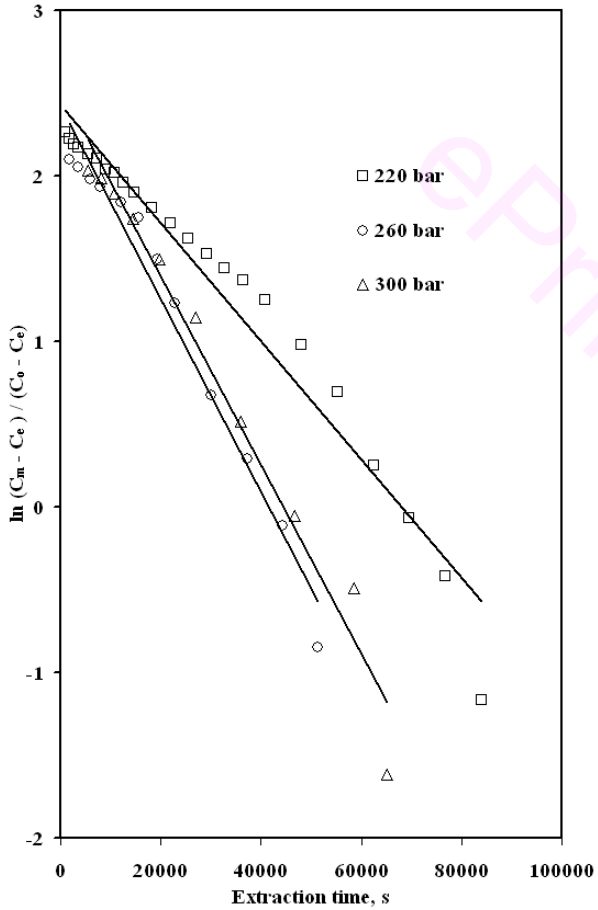


Fig. 2.7. Regression of Diffusion model to extraction data.

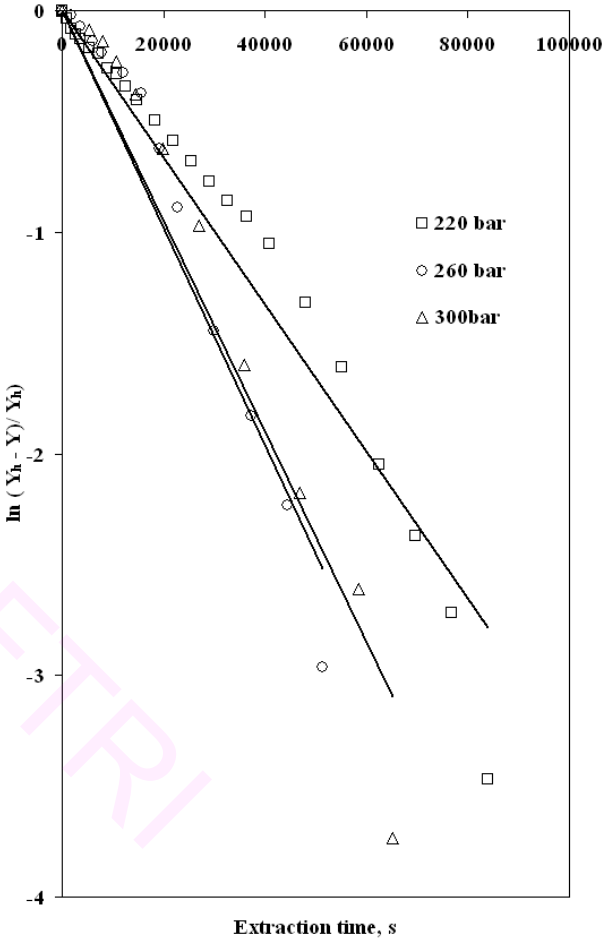


Fig. 2.8. Regression of Exponential model to extraction data.

Figure 2.10 shows isobaric experimental data and predicted extraction curves based on logistic single-component model (equation 2.9) at 313 K. The data regressed the model at all extraction pressures with high regression coefficient of 0.95 and above. The parameter of the model "b" and t_m are listed in Table. 2.7. The table also showed the average absolute relative deviation (AARD) being in the range 10% to 26% which explains that Logistic model is also suitable to predict the extraction behavior.

The physical meaning of the adjusted parameters in each model is important for the understanding and the discussion of the applicability of the models in other operational conditions. In the logistic model there are parameters whose meanings must still be defined: b and t_m. It is evident in equation 2.9 that the value of the parameter t_{mi} corresponds to the instant in which the extraction rate of each group of compounds reaches its maximum.

The same can be said of the value of t_m in the single-component Logistic model. The parameter b_i, up to now, is only an adjustable parameter. The Logistic model has been successfully applied for the supercritical CO_2 extraction of ginger [Martinez *et al*., 2003]. The values of model parameters reported therein compare well with the values found in our study implying the application of logistic model to the supercritical fluid extraction. Comparing the correlation coefficients of models, one can conclude that the Diffusion model and Logistic model are better to represent the extraction process.

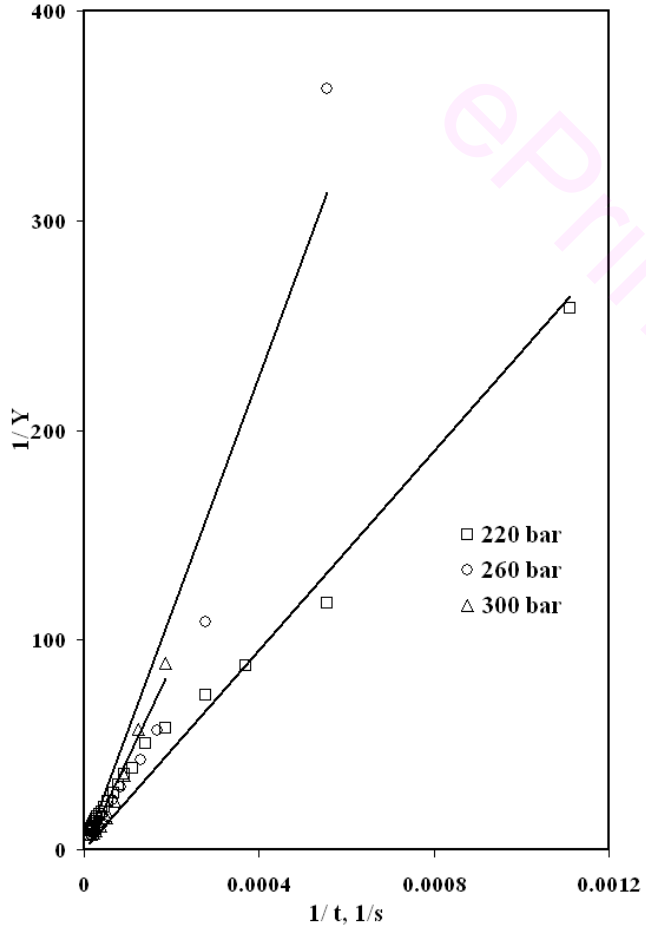


Fig. 2.9. Regression of Langmuir model to extraction data.

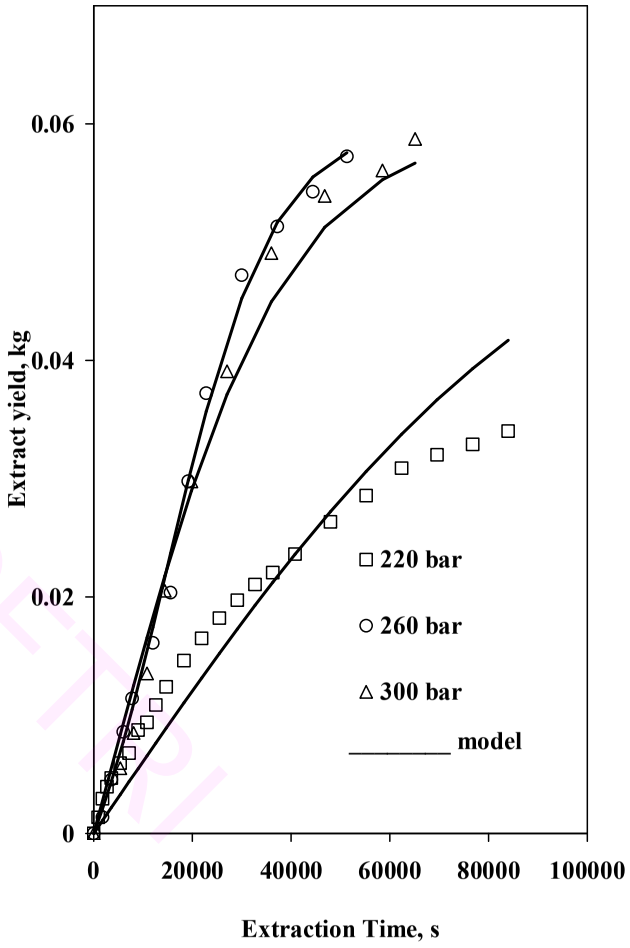


Fig. 2.10. Extract yield Vs time of extraction at various pressures.

2. 3. 2. Characterization of the extract for bioactive components

During the progress of extraction, each fraction as well as combined cumulative extract obtained by $SCCO_2$ extraction process was analyzed by HPLC to find out the composition of the components. Typical HPLC diagrams of the extract obtained under some selected process conditions (Figures 2.11 to 2.13) indicated clear separation of bakuchiol from the seeds at retention time of 22-24 min. The presence of psoralen and other minor components in the extract were also observed as different peaks at different retention times.

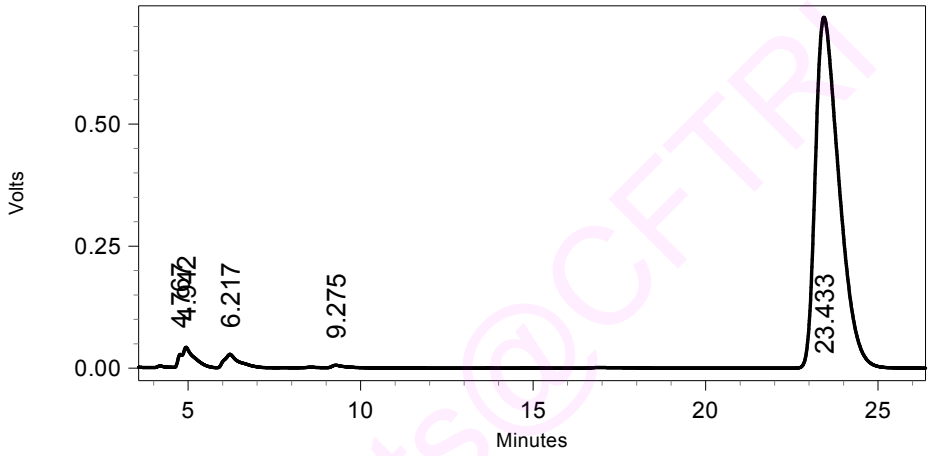


Fig. 2.11. HPLC chromatogram of the extract obtained by $SCCO_2$ at 220 bar, 40°C. Extraction period: 21900 s.

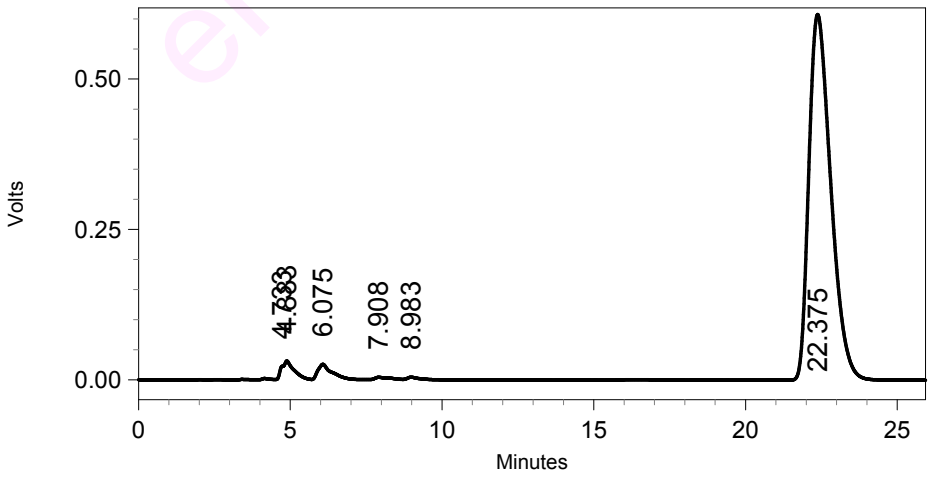


Fig. 2.12. HPLC chromatogram of the extract obtained by $SCCO_2$ at 260 bar, 40°C. Extraction period: 19800 s.

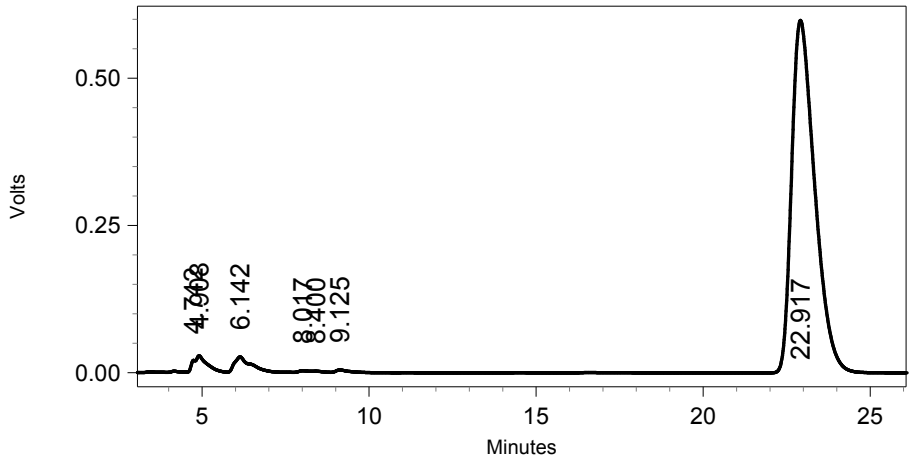


Fig. 2.13. HPLC chromatogram of the extract obtained by $SCCO_2$ at 300 bar, 40°C. Extraction period: 19200 s

The identified components of the extract obtained at 280 bar and 50°C with their retention times are given (Table. 2.9). LC-MS chromatogram of $SCCO_2$ extract is also provided (Fig. 2.14). Mass spectra data of the identified compounds in the extract are indicated in Table 2.10 and individual spectra of the identified compounds are produced in Fig. 2.15. NMR spectra data of purified compounds is shown in Table 2.11.

Table 2.9. Major compounds identified in the extract

Peak	Retention time (min.)	Compound identified
1	4.05	Psoralen
2	4.23	Isopsoralen
3	5.44	Corylin
4	5.67	Psoralidin
5	6.80	Bavachromene
6	7.51	Isobavachalcone
7	7.96	Corylifol A
8	8.52	Bavachalcone
9	24.58	Bakuchiol

The clear separation of the peaks was observed indication a total number of components as by HPLC. To identify the components, the extract was subjected to

LC-MS studies; based on mass ion as well as the fragmentation pattern, literature data, use of comparison with the data of standard samples.

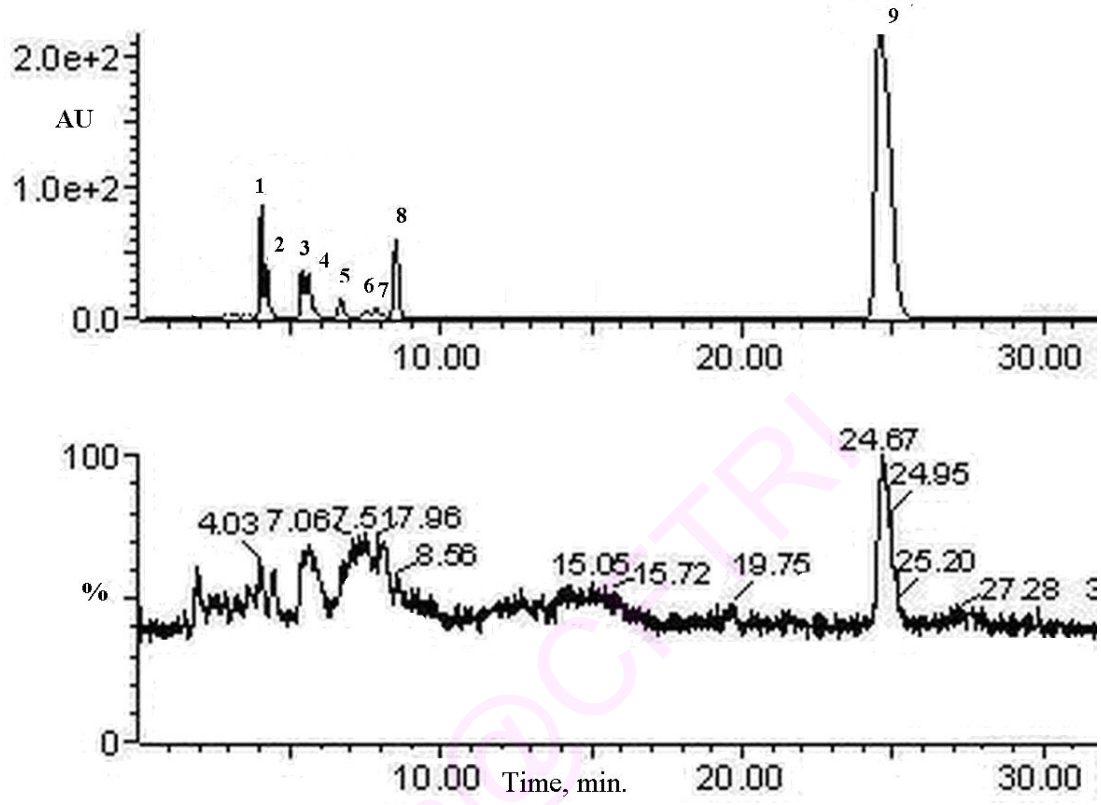


Fig. 2.14. LC-MS chromatogram of $SCCO_2$ extract

Peak 1 and 2 in ESI (-) - MS data, [M-H] at 185, so the molecular weight of this component was 186, corresponding to the mass of psoralen or isopsoralen. Comparing with the mass spectral fragmentation and retention time of standard samples, peak 1 was confirmed as psoralen and peak 2 as isopsoralen. The mass spectrum of peak 3 exhibited molecular ion peak at [M-H] 319 and according to literature data [Guo *et al*., 2005], this peak was identified as corylin. Guo *et al*., 2005 separated and identified psoralidin from *P.corylifolia*, as molecular weight was 336.10 and this matched with peak 4 in terms of MS data and [M-H] was 335, so peak 4 was identified as psoralidin. By comparing the literature mass spectral data [Guo *et al*., 2005] peak 5 was identified as bavachromene. Peak 6 showed molecular ion peak at [M-H] at 323 and this was identified as isobavachalcone. Yin *et al.*, 2004 separated and identified corylifol A from *P.corylifolia,* and the molecular weight was calculated as 390, which was very similar to ion peak on ESI (-)-MS data,[M-H] was 389 for peak 7, so this peak was tentatively identified as corylifol A. Peak 8 showed mass ion

peak at [M-H] as 337, it was identified as bavachalcone. Peak 9 was conformed as bakuchiol by comparing the mass spectrum of peak 9 with that of pure bakuchiol, the molecular ion peak was also at [M-H] at 255. The identification of the compounds was conformed by mass ions and mass fragmentation pattern comparison of the literature data and also retention times as well as the predicted data of mass spectral data by Cambridge software (http://www.cambridgesoft.com).

Pure bakuchiol showed an antioxidant activity of 1.24 mM (IC_{50} value) as against 0.029 mM for synthetic antioxidant BHA. ACE inhibitory activity of bakuchiol was 0.74 mM (IC_{50} value) compared to value of 0.071mM (IC_{50} value) obtained for synthetic enalapril.

Table 2.10. Mass spectra data of the identified compounds

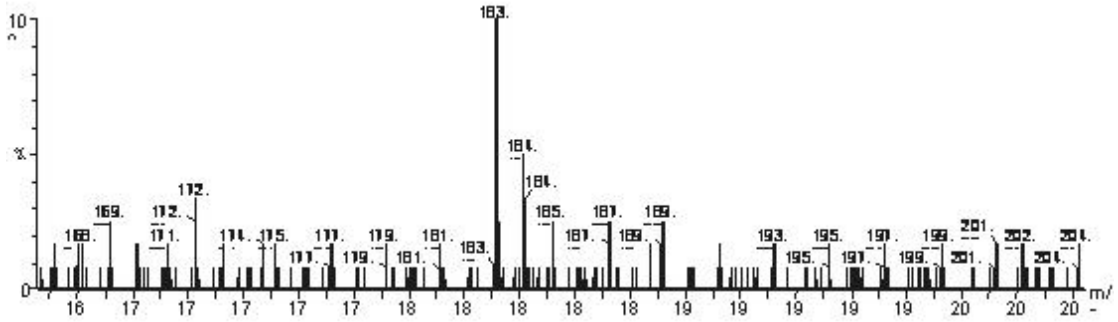
Compound	Molcular formula, Mass	Mass spectral data, m^+ % abundance
Psoralen	$C_{11}H_6O_3$, 186	183(100), 184.17(45), 185.19(25), 187.28(26) 189.20(26)
Isopsoralen	$C_{11}H_6O_3$, 186	183(100), 184.17(45), 185.19(25), 187.28(26) 189.20(26)
Corylin	$C_{20}H_{16}O_4$, 320	317.40(15), 318.36(7), 319.38(100), 319.41(50) 320.39(24), 321.36(12)
Psoralidin	$C_{20}H_{16}O_5$, 336	325.46(100), 335.42(45), 335.48(85), 335.61(54) 339.47(80), 340.53(28)
Bavachromene	$C_{20}H_{18}O_4$, 322	320.75(18), 321.34(30), 321.36(50), 321.38(100) 321.43(50), 321.49(30), 322.23(18), 322.42(32)
Isobavachalcone	$C_{20}H_{20}O_4$, 324	321.38(18), 323.43(40), 324.41(10), 325.46(100) 326.45(25)
Corylifol A	$C_{25}H_{26}O_4$, 390	375.51(10), 389.49(80), 389.52(100), 390.50(30), 391.49 (10), 401.25(10)
Bavachalcone	$C_{21}H_{22}O_4$, 338	325.45(35), 326.45(12), 333.38(20), 335.41(12), 337.46(100), 339.48(50), 339.52(40), 340.53(10)
Bakuchiol	$C_{18}H_{24}O$, 256	248.29(20), 248.31(30), 253.26(20), 253.30(30), 255.38(75), 255.42(100), 255.43(65), 255.47(40), 261.38(30).

Table 2.11. ^{1}H and ^{13}C NMR spectra data of purified compounds.

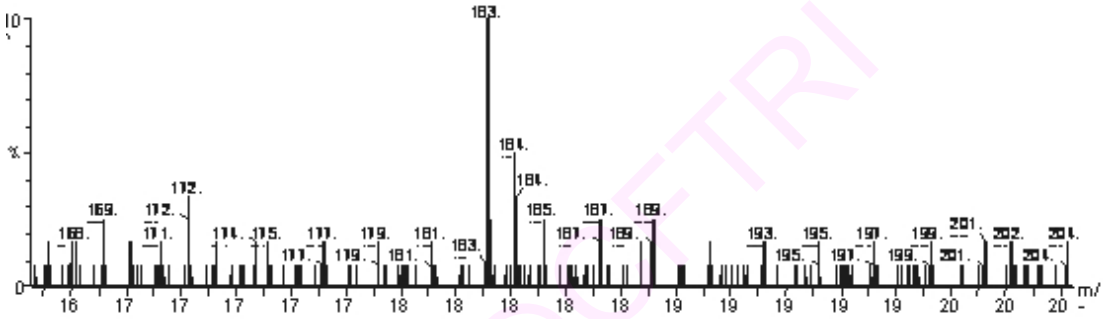
Compound	^{13}C NMR (500Hz)	^{1}H NMR (500Hz)
Bakuchiol	18.1 (C_{19}), 22.7 (C_{12}), 26.4 (C_{15}), 29.2 (C_{16}), 41.0 (C_{10}), 42.2 (C_{11}), 111.5 (C_{18}), 114.7 (C_6), 115.1 (C_2), 124.5 (C_8), 126.2 (C_{13}), 127.1 (C_4), 127.6 (C_5), 128.2 (C_9), 129.8 (C_{14}), 130.7 (C_3), 145.6 (C_{17}), 154.5 (C_1).	1.22 (s, 2H, H-10), 1.50 (d, 1H, J=6, H-16), 1.6 (s, 3H,H-15, H-19), 1.97 (d, 2H, J-9.0, H-12), 5.05 (d, 2H, J=1.0, H-18), 5.07 (OH, J=1.5, H-7), 5.13 (d, 1H, J=1.5, H-13), 5.876 (d, 1H, J=11, H-17), 6.05 (d, 1H, J=16, H-9), 6.25 (d, 1H, J=16, H-8), 6.779 (d, 2H, J=9.0, H-6, H-2), 7.247 (d, 2H, J=8.5, H-3, H-5)
Psoralen	99.556 (C_6), 106.059 (C_3), 114.362 (C_{10}), 115.118 (C_{12}), 119.512 (C_{13}), 124.563 (C_4), 143.708 (C_{11}), 146.585 (C_2), 151.753 (C_7), 156.119 (C_5), 160.650 (C_9)	6.38 (d, 1H, J=10.0,H-10), 6.85 (d, 1H, J=1.0, H-3), 7.487 (s, 1H, H-6), 7.702 (s, 1H, H-11), 7.71 (d, 1H, J=2.0, H-13), 7.809 (d, 1H, J=10.0, H-2)
Isopsoralen	160.655 (C_7), 156.119 (C_{13}), 148.201 (C_5), 146.583 (C_2), 143.707 (C_9), 124.562 (C_4), 123.497 (C_{11}), 115.1 (C_{10}), 114.362 (C_8), 108.471 (C_{12}), 106.056 (C_3)	6.406 (d, 1H, J=7, H-8), 6.847(s, 1H, H-3), 7.14 (d, 1H, J=0.5, H-12), 7.39 (d, 1H, J=8.5, H-11), 7.44 (d, 1H, J=8.5, H-9), 7.82 (d, 1H, J=9.5, H-2)

Literature reports indicated variation in number of the compounds identified compared to the compounds identified in the present study. A meroterpene and four flavonoids were isolated from the seeds of *P. corylifolia* as antioxidative components. Their structures were elucidated by spectral data and identified as bakuchiol, bavachinin, bavachin, isobavachin and isobavachalcone [Haraguchi *et al*., 2002]. Three new flavonoids corylifols, along with thirteen known compounds isolated from the seeds of *P.corylifolia*.L have been reported [Yin *et al.,* 2004]. The occurrence of six compounds, bakuchiol, psoralen, isopsoralen, corylifolin, corylin and psoralidin in the extract were earlier reported as isolated and identified from the herb *P.corylifolia*.L [Jiangning *et al.,* 2005]. Research has been carried out to study the effect of geographical regions on the components of *P. corylifolia* [Zhao *et al.*, 2005]. The variation in identified compounds may be attributed to geographic source of the plant material and extraction methods used. In an elaborate study [Chen *et al*., 2005], more than 188 compounds have been deducted in *P.corylifolia* by integration of various

techniques such as ion-exchange chromatography fractionation, reversed-phase liquid chromatography, atmospheric pressure chemical ionization mass spectrometer and matrix-assisted laser desorption/ionization time-of-flight mass.

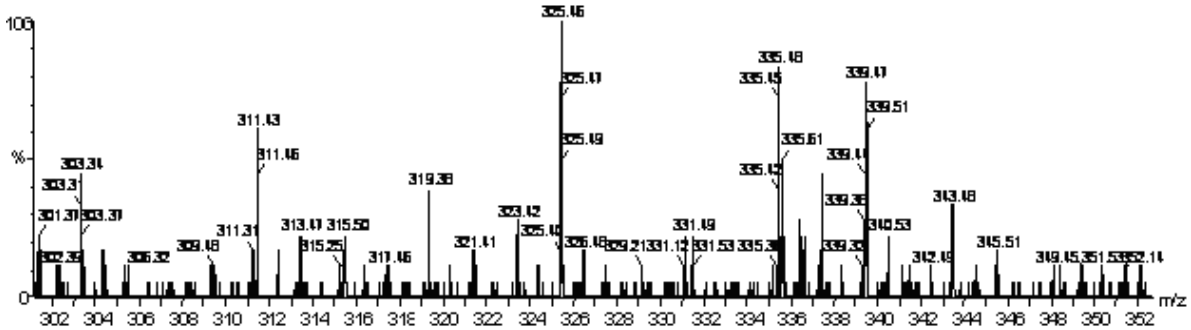


Psoralen



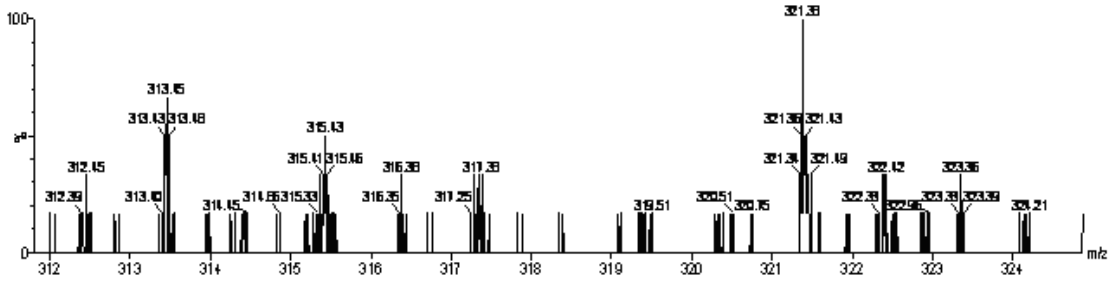
IsoPsoralen

Corylin

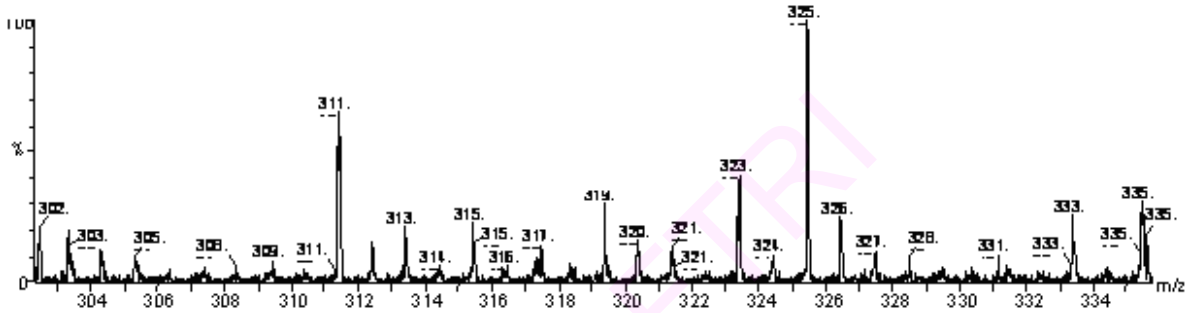


Psoralidin

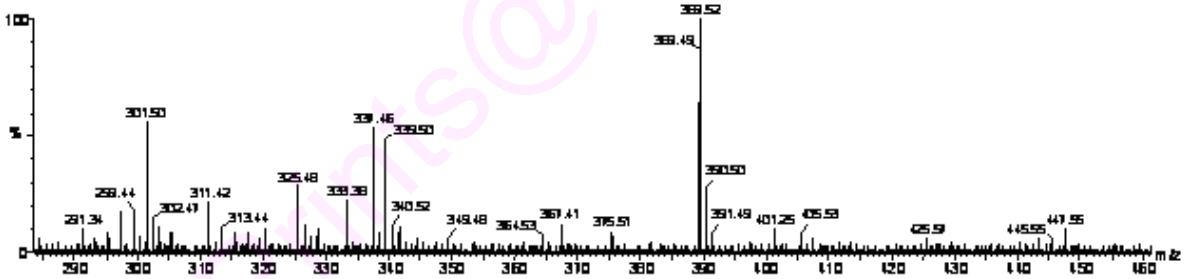
Fig. 2.15. Mass spectra of the individual compounds.



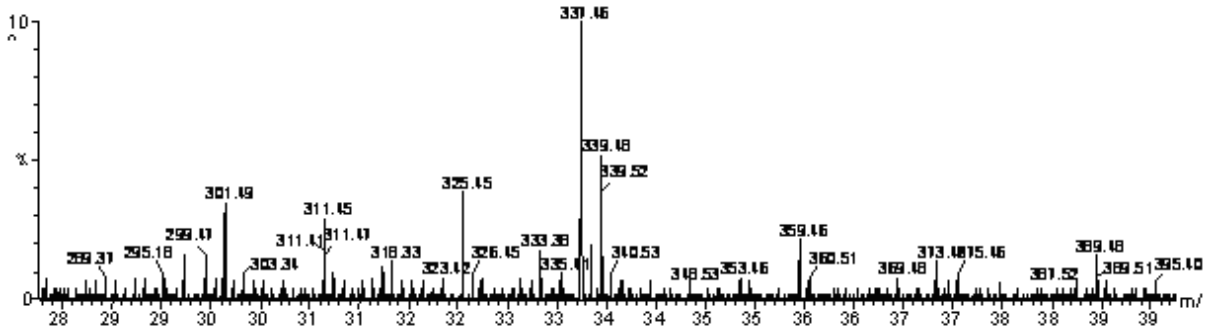
Bavachromene



IsoBavachromene

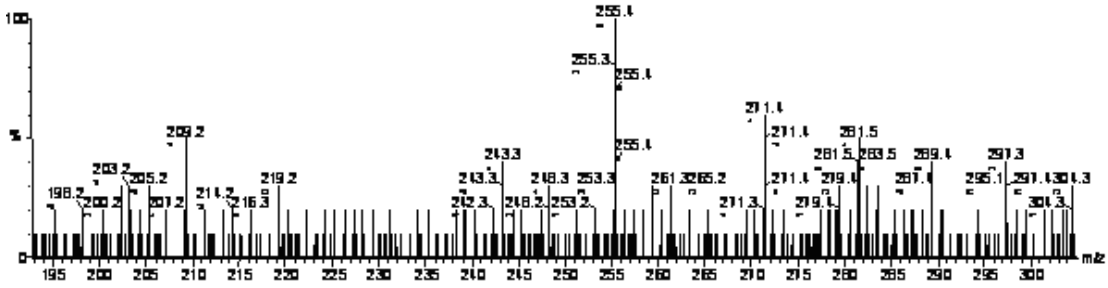


Corylifol A



Bavachalcone

Fig. 2.15. Mass spectra of the individual compounds (continued).



Bakuchiol

Fig. 2.15. Mass spectra of the individual compounds (continued).

2.3.3. Prediction of critical properties

Table 2.12 to Table 2.15 give the values of group contributions for both structures of bakuchiol and psoralen as per Ambrose, Joback, Klincewicz-Reid and Lyderson method which help in calculating critical temperature, critical pressure, critical volume and normal boiling point. On substituting these values suitably in equations 2.1 to 2.13, values of critical temperature, critical pressure, critical volume and normal boiling point of bakuchiol and psoralen were obtained. Table 2.16 gives comparative analysis of predicted critical properties of bakuchiol and psoralen using various prediction methods. It can be concluded from the Table 2.16 that various prediction methods gave almost similar results for bakuchiol. Klincewicz-Reid simplified method, which does not require group contributions values, predicted high deviations for properties among all prediction methods compared to predictions by CHEMDRAW® software. Joback method predicted very high critical pressure for psoralen. It must be born in mind, Joback method is the only procedure for calculating normal boiling point. Predicted values for critical properties by four methods deviated 0.2% to 21.9% from the prediction given by CHEMDRAW® software.

Some of the selected high molecular weight bioactive components and their critical properties available in literature along with that predicted for bakuchiol (predicted by Joback method) are listed in Table 2.17. It is seen that there is big difference in values of critical properties related to carotene in literature. Hence, one has to be very cautious in applying the property data obtained in this work to real systems as there could be a big difference between predicted and experimental values.

Predicted vapor pressures as a function of temperature using Riedel method (equation 2.23) and Edalat method (equation 2.30) for both bakuchiol and psoralen is

in Figure 2.16. Both the methods were in good agreement in estimating the vapor pressure. At a temperature of 403 K, the predicted vapor pressure of bakuchiol was $1x10^{-8}$ mm Hg. while it was 1.836 mm Hg. for psoralen. It is apparent that there is substantial difference between the vapor pressure of bakuchiol and psoralen which may play an important role if one desires separation of bakuchiol from psoralen by distillation methods.

Table 2.12. Group contributions values of bakuchiol and psoralen as per Ambrose method.

Bakuchiol

Group		ΔT_{cl}	ΔP_{cl}	ΔV_{cl}	$\Sigma\Delta T_{cl}$	$\Sigma\Delta P_{cl}$	$\Sigma\Delta V_{cl}$
-CH3	3	0.138	0.226	55.1	0.414	0.678	165.3
>CH2	2	0.138	0.226	55.1	0.276	0.452	110.2
=CH-	4	0.113	0.194	45.1	0.452	0.774	180.4
=CH2	1	0.113	0.194	45.1	0.113	0.194	45.1
=CH- (ring)	4	0.075	0.15	37	0.3	0.598	148
>C<	1	0.018	0.196	38.1	0.018	0.196	38.1
=C<	1	0.07	0.15	37.1	0.07	0.15	37.1
=C< (ring)	2	0.075	0.188	37	0.15	0.375	74
=-OH (phenol)	1	0.198	-0.025	15	0.198	-0.025	15
					1.991	3.391	813.2

Psoralen

Group		ΔT_{cl}	ΔP_{cl}	ΔV_{cl}	$\Sigma\Delta T_{cl}$	$\Sigma\Delta P_{cl}$	$\Sigma\Delta V_{cl}$
-O- ring	2	0.09	0.16	10	0.18	0.32	20
=O	1	0.09	0.16	10	0.09	0.16	10
=CH- (ring)	6	0.075	0.15	37	0.45	0.897	222
=C< (ring)	5	0.075	0.15	37	0.375	0.748	185
					1.095	2.125	437

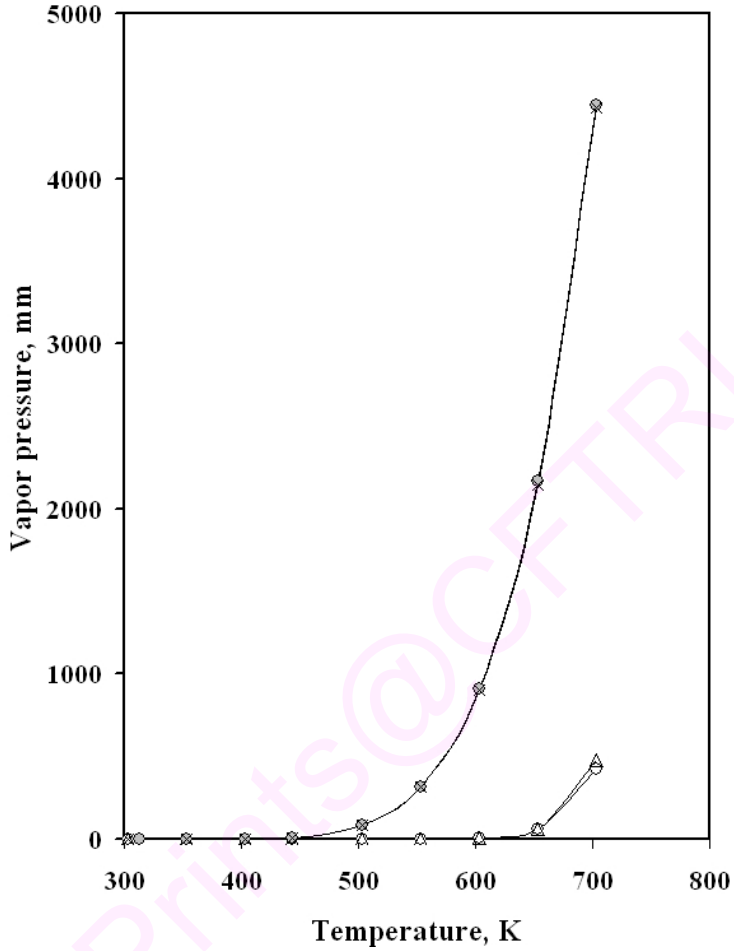


Fig. 2.16. Predicted vapor pressure as a function of temperature.

-o- Bakuchiol-1; - Δ- Bakuchiol-2 ; - ●- Psoralen -1; - x - Psoralen- 2
1 following component name indicates prediction by Riedel method;
2 following component name indicates prediction by Edalat method.

Table 2.13 . Group contributions values of bakuchiol and psoralen as per Joback method.

Bakuchiol:

Group		ΔTbj	ΔTcj	ΔPcj	ΔVcj	ΣΔTbj	ΣΔTcj	ΣΔPcj	ΣΔVcj
-CH3	3	23.58	0.0141	-0.001	65	70.74	0.0423	-0.004	195
-CH2-	3	22.88	0.0189	0	56	68.64	0.0567	0	168
>C<	1	18.25	0.0067	0.0043	27	18.25	0.0067	0.0043	27
=CH-	4	24.96	0.0129	-6E-04	46	99.84	0.0516	-0.002	184
>C=	1	24.14	0.0117	0.0011	38	24.14	0.0117	0.0011	38
=CH- (ring)	4	26.73	0.0082	0.0011	41	106.9	0.0328	0.0044	164
=C< (ring)	2	31.01	0.0143	0.0008	32	62.02	0.0286	0.0016	64
=-OH (phenol)	1	76.34	0.024	0.0184	-25	76.34	0.024	0.0184	-25
						526.9	0.2544	0.0238	815

Psoralen:

Group		ΔTbj	ΔTcj	ΔPcj	ΔVcj	ΣΔTbj	ΣΔTcj	ΣΔPcj	ΣΔVcj
-O- ring	2	31.22	0.0098	0.0048	13	62.44	0.0196	0.0096	26
=O	1	-10.5	0.0189	0.0101	36	-10.5	0.0189	0.0101	36
=CH- (ring)	6	26.73	0.0082	0.0011	41	160.4	0.0492	0.0066	246
=C< (ring)	5	31.01	0.0143	0.0008	32	155.1	0.0715	0.004	160
						367.4	0.1592	0.0303	468

Table 2.14. Group contributions values of bakuchiol and psoralen as per Klincewicz-Reid method.

Bakuchiol

Group	Nos.	ΔT_{cl}	ΔP_{cl}	ΔV_{cl}	$\Sigma\Delta T_{cl}$	$\Sigma\Delta P_{cl}$	$\Sigma\Delta V_{cl}$
-CH3	3	-2.433	0.026	16.2	-7.299	0.078	48.6
>CH2	2	0.353	-0.015	16.1	0.706	-0.03	32.2
=CH-	4	3.786	-0.05	9.8	15.14	-0.2	39.2
=CH2	1	-0.991	-0.015	13.9	-0.991	-0.015	13.9
=CH- (ring)	4	3.373	-0.066	5.1	13.49	-0.264	20.4
>C<	1	16.42	-0.136	8.95	16.42	-0.136	8.95
=C<	**1**	7.169	-0.067	2.7	7.169	-0.067	2.7
=C< (ring)	2	5.623	-0.089	0.2	11.25	-0.178	0.4
=-OH (phenol)	1	-28.93	-0.19	-24	-28.93	-0.19	-24
					26.95	-1.002	142.4

Psoralen

Group		ΔT_{cl}	ΔP_{cl}	ΔV_{cl}	$\Sigma\Delta T_{cl}$	$\Sigma\Delta P_{cl}$	$\Sigma\Delta V_{cl}$
-O- ring	2	7.127	-0.116	-36.6	14.25	-0.232	-73.2
=O	1	0	0	0	0	0	0
=CH- (ring)	6	3.373	-0.066	5.1	20.24	-0.396	30.6
=C< (ring)	5	5.623	-0.089	0.2	28.12	-0.445	1
					62.61	-1.073	-41.6

Table 2.15. Group contributions values of bakuchiol and psoralen as per Lyderson method.

Bakuchiol

Group		ΔT_{cl}	ΔP_{cl}	ΔV_{cl}	$\Sigma\Delta T_{cl}$	$\Sigma\Delta P_{cl}$	$\Sigma\Delta V_{cl}$
-CH3	3	0.02	0.227	55	0.06	0.681	165
>CH2	2	0.02	0.227	55	0.04	0.454	110
=CH-	4	0.018	0.198	36	0.072	0.792	144
=CH2	1	0.018	0.198	45	0.018	0.198	45
=CH- (ring)	4	0.011	0.154	37	0.044	0.616	148
>C<	1	0	0.21	41	0	0.21	41
=C<	1	0	0.198	36	0	0.198	36
=C< (ring)	2	0.011	0.154	36	0.022	0.308	72
-OH (phenol)	1	0.035	-0.02	3	0.035	-0.02	3
					0.291	3.437	764

Psoralen

Group		ΔT_{cl}	ΔP_{cl}	ΔV_{cl}	$\Sigma\Delta T_{cl}$	$\Sigma\Delta P_{cl}$	$\Sigma\Delta V_{cl}$
-O- ring	2	0.014	0.12	8	0.028	0.24	16
=O	1	0.02	0.12	11	0.02	0.12	11
=CH- (ring)	6	0.011	0.154	37	0.066	0.924	222
=C< (ring)	5	0.011	0.154	36	0.055	0.77	180
					0.169	2.054	429

Table 2.16. Comparison of predicted critical properties of bakuchiol and psoralen between different prediction methods.

Property	Ambrose	Joback	Kleincewicz-Reid[a]	Kleincewicz-Reid[b]	Lyderson	Chemdraw®
Bakuchiol:						
T_c, K	943	947.9	991.8	1025	931.6	821.4
P_c, bar	18	19.4	21.7	21.2	18	18.97
V_c,cc/gmol	853	832.5	884.4	618.7	804	882.5
T_B, K	--	724.9	--		--	720.4
Psoralene:						
T_c, K	850	793.7	886.9	859.3	841	881.4
P_c, bar	31	46.5	36.8	32.2	32.5	38.15
V_c,cc/gmol	477	485.5	504.4	304.4	469	486.5
T_B, K	--	565.4	--	--	--	594.9

*CHEMDRAW® Ultra version 8.0 (Cambridgesoft Corporation, Cambridge, USA)

Kleincewicz-Reid[a] prediction by group contribution.

Kleincewicz-Reid[b] prediction by simplified equation.

Table 2.17. Critical properties of some selected bioactives.

Compound	Molecular Wt.	T_c, K	P_c, bar	T_b, K	V_c, L/mol
cholesterol[a]	386.65	778.7	12.2	764.8	0.362
carotene[a]	536.87	801	8.1	-	0.537
carotene[b]	536.87	1485.6	8.0	1209.4	1.846
tocopherol[c]	430.72	861.9	10.2	703.8	-
bakuchiol[d]	256.38	948	19.4	724.9	0.832

[a] Hartono *et al*., 2001; [b] Skerget and Knez, 1997;
[c] Basalingappa et al., 2001; [d] present study.

2.3.4. Solubility prediction

Values of experimental and predicted solubilities of bakuchiol and psoralen based on PR-EOS and SRK-EOS along with enhancement factors for bakuchiol and

psoralen are given in Table 2.18. The solubilities for both bakuchiol and psoralen are plotted in Figure 2.17. The solubility (in terms of mole fraction) of both bakuchiol and psoralen were found in the range 10^{-6} to 10^{-4} and the solubility increased with pressure. Whereas average absolute relative deviation (AARD) for prediction of solubility of bakuchiol was much high, AARD was less for psoralen. Enhancement factors for bakuchiol were found to be much higher compared to that of psoralen. This is due to the fact that bakuchiol deviates from ideal gas behavior much more than that of psoralen. When the extract is considered as a single pseudo component and the critical properties of pseudo-component is based on average value of critical properties of bakuchiol and psoralen, the predicted solubility was much better in agreement with experimental value of solubilities (Table 2.18).

Negative k_{ij} values of up to -1.43 for unsaturated carboxylic acids have been reported in literature [Abbott *et al*., 2005]. In the present study, negative values of k_{ij} up to -1.38 were reported for bakuchiol and psoralen by both PR-EOS and SRK-EOS.

High values of enhancement factor up to 10^7 have also been reported for several systems studied using PR and SRK equation of state [Bae *et al*., 1987]. The present study also reported high enhancement factors for the bakuchiol (7.8×10^{16} to 2.3×10^{18}) and psoralen (3.3×10^3 to 1×10^4) indicating high non-ideal behavior of these compounds. When the extract was considered to be a single pseudo-component, enhancement factors were in the range 1.6×10^4 to 6.6×10^4.

2.4. Conclusions

Critical properties like critical temperature, critical pressure, critical volume were predicted for bakuchiol and psoralen based on 4 different group contribution methods. The solubility of bakuchiol and psoralen at 220, 260 and 300 bar was predicted based on equation of state models Peng-Robinson and SRK. The models indicated high enhancement factors for both the components implying high non-ideal behavior. Even though the yield of extract in SCFE process was less than that obtained by solvent extraction method, bakuchiol content in the extract obtained by SCFE process was substantially higher that of solvent extraction process. Maximum bakuchiol content of 51% in the extract was obtained by SCFE at a pressure of 300 bar. Diffusion model and Logistic model are better to represent the extraction process.

The extract was characterized for bioactive components by HPLC, LC-MS, mass spectra and NMR and the major components namely psoralen, isopsoralen, corylin, psoralidin, bavachromene, isobavachalcone, corylifol-A, bavachalcone and bakuchiol were identified and bakuchiol was found to be the major component in the extract. Bakuchiol showed clear antioxidant activity and ACE inhibition activity measured in terms of IC_{50} values.

Table 2.18. Experimental solubility, predicted solubility and enhancement factor for the extract and its key components by PR-EOS and SRK-EOS.

a. Solubilities

Component	Pressure, bar			AARD,%
	220	260	300	
Bakuchiol-Expt	8.59E-05	1.03E-04	1.55E-04	
Bakuchiol-PR	7.08E-06	3.60E-05	1.55E-04	52.3
Bakuchiol-SRK	7.86E-06	3.83E-05	1.55E-04	51.2
Psoralen-Expt	1.18E-04	1.42E-04	2.13E-04	
Psoralen-PR	9.26E-05	1.42E-04	2.19E-04	8.2
Psoralen-SRK	9.63E-05	1.43E-04	2.13E-04	6.5
Extract-Expt	3.06E-04	3.68E-04	5.51E-04	
Extract-PR	2.16E-04	3.68E-04	6.59E-04	16.3
Extract-SRK	2.27E-04	3.68E-04	5.89E-04	10.9
b. Enhancement factor:				
Psoralen -PR	3.40E+03	6.15E+03	1.09E+04	
Psoralen -SRK	3.53E+03	6.20E+03	1.06E+04	
Bakuchiol-PR	7.79E+16	4.67E+17	2.32E+18	
Bakuchiol-SRK	8.65E+16	5.00E+17	2.32E+18	
Extract-PR	1.59E+04	3.19E+04	6.59E+04	
Extract-SRK	1.67E+04	3.19E+04	5.89E+04	

c. Interaction parameter k_{ij}:	
Extract - PR	0.516
Extract-SRK	-1.127
Bakuchiol-PR	-0.113
Bakuchiol-SRK	-0.400
Psoralen-PR	0.398
Psoralen-SRK	-1.384

Note: Extract-PR and Extract-SRK denote the extract when considered as single pseudo-component.

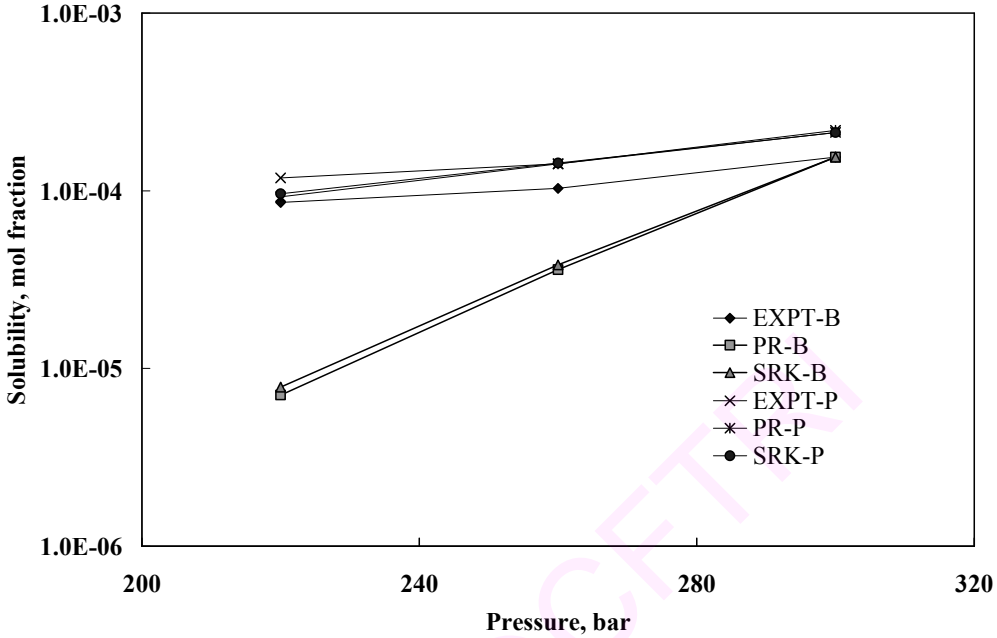


Fig. 2.17. Experimental and predicted Solubility of key components Vs pressure by PR-EOS and SRK-EOS. B in the legend end denotes bakuchiol; P in the legend end denotes psoralen.

2.5. Nomenclature

a	Intermolecular attraction parameter
b	Co volume parameter
C	Concentration of the solute in mole fraction (equation 2.1)
D_{aX}	Diffusion coefficient in the solid phase (equation 2.8)
D_{aY}	Axial dispersion coefficient (equation 2.7)
D_e	Effective diffusion coefficient or diffusivity (equation 2.1)
E	Enhancement factor
f_2^s	Fugacity of solute in the solid phase;
f_2^v	Fugacity of solute in the gas phase
h	Axial coordinate in the extraction bed (equation 2.7)
J(x,y)	Interfacial mass transfer flux (equation 2.7 and 2.8)
k_E	Constant in exponential model (equation 2.4)
k_{ij} and n_{ij}	Adjustable binary interaction parameters
k_L	Constant in Langmuir's adsorption model (equation 2.5)
m_t	Adjustable parameter in logistic model (equation 2.9)
M_w	Molecular weight of the compound
n	Amount of substance (in moles)
n_A	Number of atoms in the compound
P	Pressure
P_c	Critical pressure
P^{sat}	Vapor pressure
P^s_r	Reduced vapor pressure (P^s/P_c).
R	Universal gas constant
r	Radius of diffusion (equation 2.2)
R	Maximum radius of diffusion
S_1,S_2,S_3, S_4,S_{21}, S_{22}	Constants defined in equations (2.24) to (2.29)
T	Temperature
t	Extraction time
T_B	Normal boiling temperature
T_c	Critical temperature
t_m	Adjustable parameter in logistic model (equation 2.9)

T_r	Reduced temperature
T_{rb}	Reduced temperature at normal boiling temperature (T_B/ T_C)
u	Interstitial velocity of the solvent (equation 2.7)
V	Volume
X	Solute mass ratios in the solid phases (equation 2.8)
Y	Extraction yield in Langmuir adsorption model (equation 2.5)
Y	Solute mass ratios in the fluid phases (equation 2.7)
y_{cal} and y_{exp}	Calculated and experimental solubilities of solute
Y_D	Ratio between extract concentration at time, t and the initial extract concentration of the matrix (equation 2.2)
Y_f	Yield at infinite extraction time (equation 2.5)
Y_h	Highest yield in weight percent (equation 2.4)
ΔB_J	Group Contributions for boiling point
ΔP_A	Group Contributions for pressure in Ambrose equation (2.14)
ΔP_J	Group Contributions for pressure in Joback equation (2.15).
ΔP_K	Group Contributions for pressure in Klincewicz-Reid equation (2.16).
ΔP_L	Group Contributions for pressure in Lydersen equation (2.17).
ΔT_A	Group Contributions for temperature in Ambrose equation (2.10)
ΔT_J	Group Contributions for temperature in Joback equation (2.11)
ΔT_K	Group Contributions for temperature in Klincewicz-Reid equation (2.12)
ΔT_L	Group Contributions for temperature in Lydersen equation (2.13)
ΔV_A	Group Contributions for volume in Ambrose equation (2.18)
ΔV_J	Group Contributions in Joback equation (2.19)
ΔV_K	Group Contributions for volume in Klincewicz-Reid equation (2.20)
ΔV_L	Group Contributions for volume in Lydersen equation (2.21)
ε	Bed porosity (equation 2.7 and 2.8)
η_1, η_2,η_3 , η_4	Constants defined in equation in (2.30) to (2.34).
ρ	Solvent density (equation 2.8)
ρ_s	Solid density (equation 2.8)
σ_A	Constant in equation 2.10.
φ	Fugacity coefficient;
ω	Acentric factor (equation 2.36)

CHAPTER 3

Enrichment of bakuchiol using Molecular distillation Technique

3.0. Introduction

The molecular distillation is suitable for the separation of a specific component from a mixture of high molecular weight compounds, provided there is significant difference in their vapor pressure (relative volatility) so as to achieve a good separation. The distance between the evaporation and condensation surface in the molecular distillation unit is very small and the operating pressure is very low (less than 0.1 Pa) leading to evaporation taking place at low temperatures that do not degrade the heat sensitive materials. As a general rule for organic compounds, each order of magnitude decrease in pressure results in a 25°C lowering of boiling temperature. This is particularly very important for heat sensitive materials. While ordinary vacuum distillations are carried out usually at 1-10 mm Hg, molecular distillations are generally conducted in the absolute pressure of 1-7 μm Hg [Gruenwedel and Whitaker, 1987]. Unlike the conventional methods that use organic solvents for separation and purification of heat sensitive components, molecular distillation completely avoids using any organic solvents in the purification/separation process [Lutisan *et al*., 2002]. Hence, molecular distillation technique scores well as an environmental-friendly process than other methods that use organic solvents that are toxic and carcinogenic. The molecular distillation has received more attention in the fat and oil industry than in any other industries.

Figure 3.1 gives schematic diagram of simple molecular distillation system. Unlike conventional evaporators, the main feature of molecular distillation unit is that the condenser is placed inside the evaporating chamber. This makes it possible to attain very low pressure (high vacuum) in the system thereby making the material to evaporate at low temperature. Therefore, the residence time in the heated region is very short making fouling/thermal damage of the product minimized. Figure 3.2 describes the evaporation taking place inside the evaporation body.

Recent research studies clearly show that the actual degree of separation achieved in a molecular distillation process depends not only on relative volatility of the components in the mixture but also on various operating process parameters. The reduction of the pressure of the space surrounding the liquid to be evaporated results in the molecules having to overcome a lower pressure in their evaporation step and

the energy required is minimal. Rate of evaporation of molecules in molecular distillation is deduced in theoretical terms based on Maxwell-Boltzmann distribution of velocities and is as below [Hollo *et al.*, 1971]:

$$N = A \tau P_g / (2 \pi M_w R_g T)^{1/2}$$

Where N = Number of moles of vapor condensing on the surface; A = unit area; τ = time; P_g = vapor pressure of the pure material; M_w = Molecular weight; R_g = universal gas constant; T = absolute temperature. The important operating factors that influence the separation of the molecules in the system are pressure (vacuum), Heating and condenser temperature, speed of wiper unit.

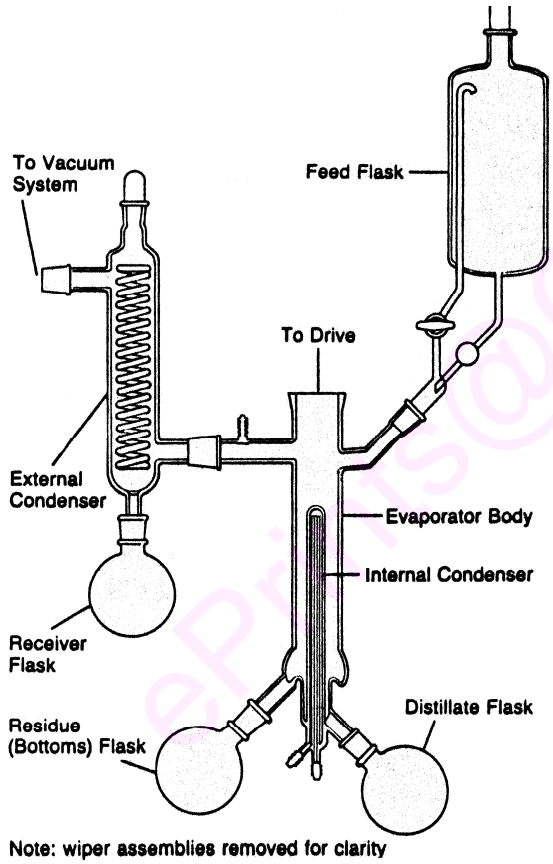


Fig. 3.1. Schematic diagram of the molecular distillation unit

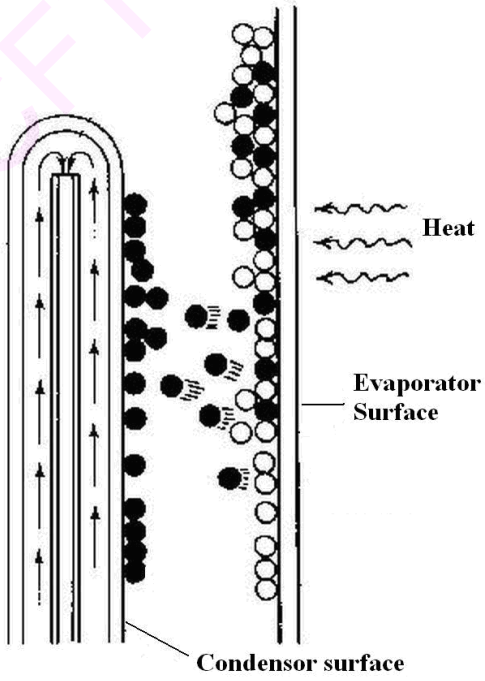


Fig. 3.2. Cross section of evaporator body. O – unevaporated molecules. ● – condensing molecules.

3.1. Literature survey on molecular distillation

Applications of high vacuum distillation to fish oils [Hickmann, 1937; Farmer, 1938; Komori, 1951], oils of plant sources [Embree, 1941], sterols [Hickman, 1940] and. brain phospholipids [Schwirth, 1943] have been studied several decades back.

Recently, there has been increased number of reports on application of molecular distillation to highly valuable bio-active components. There are several reported applications of molecular distillation found in literature: monoacylglycerols [Szelag and Zwierzykowski, 1983], fractionation polyunsaturated fatty acids from fish oils [Breivik *et al*., 1997], recovery of carotenoids from palm oil [Batistella and Maciel, 1998], fractionation of squalene from Amaranth seed oil [Sun *et al*., 1997], reduction of cholesterol in butter and lard [Lanzani *et al*., 1994, purification of sugar ester [Redmann *et al*., 1995]. Purification of structured lipids from rapeseed oil and capric acid and deodourization of structured lipids has been reported [Xu *et al*., 2002].

Separation of meadowfoam fatty acids using centrifugal molecular distillation unit has been reported [Cermak and Isbell, 2002]. Molecular distillation has been applied for the separation of free fatty acids and tocopherols from soybean oil deodorizer distillate [Shimada *et al*., 2000; Ito *et al*., 2006; Martins *et al*., 2006] and rapeseed oil deodorizer distillate [Jiang et al., 2006 and]. Purification of octacosanol from rice bran wax by molecular distillation [Chen *et al*., 2007] and optimization of process conditions has been carried out [Chen *et al*., 2005].

Modeling on the molecular distillation have been done by several authors (Ribeiro and Andrade, 2002; Cvengros *et al*., 2000). Effect of evaporation rate on purification and separation factor of molecular distillation has been studied [Kawala and Stephan, 1989; Nguyen and LeGoffic, 1997]. Heat and mass transfer in the evaporating film of a molecular evaporator has been studied [Lutisan *et al*., 2002]. Influence of operating variables of molecular distillation on deacidification of grape seed oil using the response surface methodology has been carried out [Martinello *et al*., 2007]. Prior to this, liquid phase transfer processes in high vacuum distillation have been well discussed [Bhandarkar and Ferron, 1988; Bose and Palmer, 1984]. Influences of feed and condenser temperature on the efficiency of molecular distillation have been discussed [Cvengros et al., 2000; Shao *et al.*, 2007]. Temperature intervals of main components of natural oils during molecular distillation are provided in Table 3.1.

Table 3.1. Main components and their distilling temperature intervals

Distillation interval or its main component	Temperature interval, °C	Components generally distilling in the interval	Molecular weight interval
Acid interval	50-140	Fatty acids, unsaponifiable materials of low molecular wt.	150-300
Unsaponifiable matter	140-190	Sterols	
		Vitamins	
		Dyes	300-600
		Monoglycerides	
		Diglycerides	
		Wax alcohols	
Triglyceride interval	Above 190	Triglycerides	
		Sterol esters	
		Vitamin esters	600-900
		Resins	
		Waxes	

Source: Hollo *et al*., 1971.

From the Table 3.1, one can roughly estimate the temperature at which the component of interest is likely to distill. Figure 3.3 illustrates the feasibility of separation of free fatty acid like palmitic, stearic and oleic acid and tocopherol from triglycerides in view of the substantial difference of vapor pressure among them.

Effect of non-volatile components and thermal decomposition: Non-volatile components concentrate as distillation proceeds, cover part of the active evaporation surface and so reduce its area. The initial studies on thermal damage on materials in distillation have been studied by Hickman and his co-workers [Hickman and Embree, 1948]. The degree and rate of decomposition depend not only on distilland but also on the distillation conditions particularly on the exposure time of materials in the apparatus. Decomposition hazard is defined as the product of exposure time and pressure of permanent gas [Hickman and Embree, 1948].

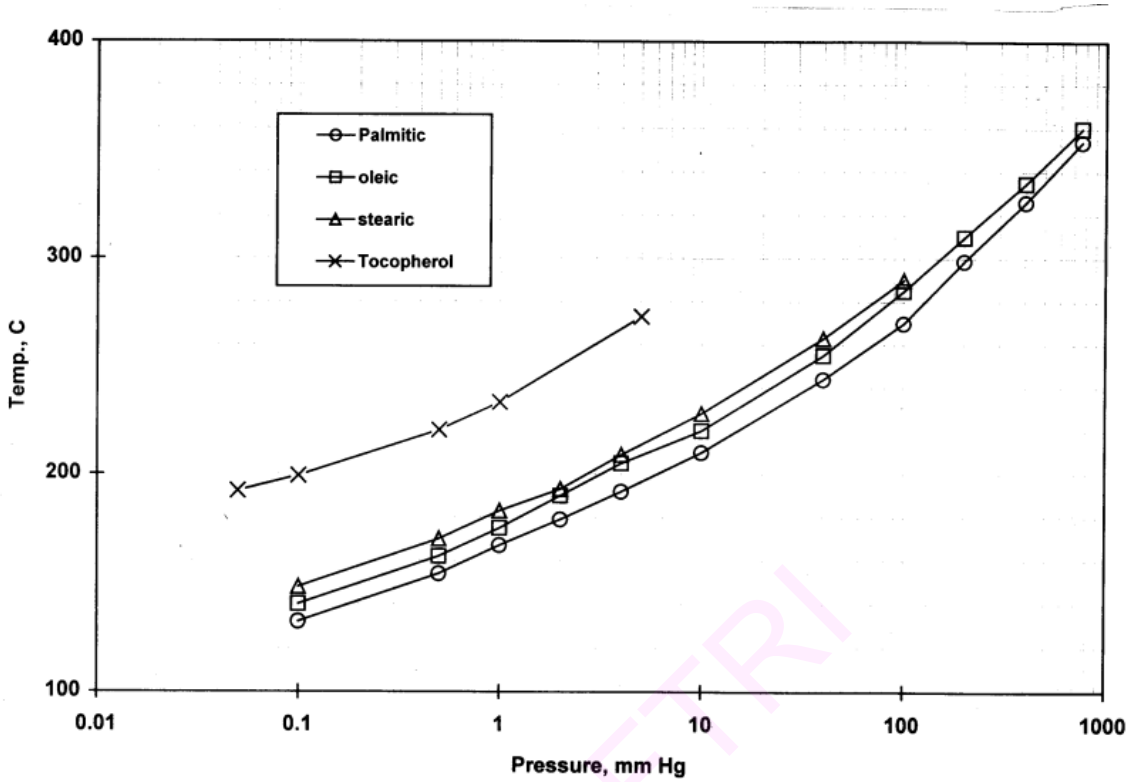


Fig. 3.3. Vapor pressure-Temperature curves for selected heat sensitive high molecular compounds.

3.1.1. Optimization of process conditions

The main objective of experimental design is to plan experiments so as in order to study the effects of certain factors/ process variables on some specific output variables and to identify the important influential factors with a given level of statistical confidence. The effects of the factors and their interactions are evaluated by computing statistical F-values, which are the ratio of effect-mean-square vs. error-mean-square. Response-surface methodology (RSM) is a modeling technique to illustrate the effects of factors on response surfaces (output variables) for the purpose of locating the optimum. Therefore, the RSM consists of the techniques for experimental design, regression analysis, and computation of the optimum conditions. There are many books that detail various aspects of design of experiments and response surface analysis [Montgomery, 1991; Akhnazarova and Kafarov, 1982]. RSM is an efficient statistical technique for optimization of multiple variables to predict best performance conditions with minimum number of experiments.

In the present study, RSM has been employed to analyze the effect of process variables such as temperature, wiper speed and pressure on the enrichment of bakuchiol content in the extracts of *Psoralea corylifolia* seeds obtained by supercritical carbon dioxide ($SCCO_2$). The optimization of process conditions for molecular distillation technique has been carried out based on RSM.

3.2. Material and Methods

3.2.1. Materials

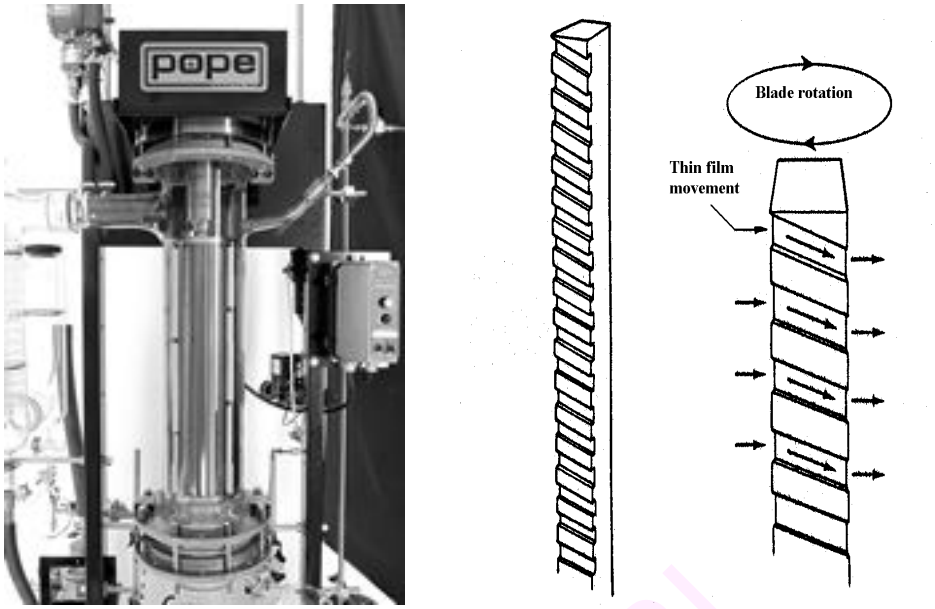
The *P.corylifolia* seed powder is a gift from M/s Kumar Organic Production, Bangalore, India. Hexane (LR grade), Acetonitrile (HPLC grade) are obtained from Merck (India) Ltd., Mumbai, India; and Toluene (Analytical Grade) from M/s Qualigens Mumbai, India. Carbon dioxide (food grade) is obtained from approved local dealer (M/s Kiran Corporation, Mysore, India).

3.2.2. *P.corylifolia* seed extract

The powdered seed samples are extracted on a pilot scale in high pressure equipment (M/s NOVA Swiss Werke AG, Switzerland) designed for working pressures of up to 1000 bar and temperature up to 100°C. The detailed operating procedure of the high pressure system has been outlined in the previous chapter 2.2.4. Pressure and temperature for the extraction are 280 bar and 50°C respectively.

3.2.3. Molecular distillation

The pilot plant molecular distillation unit (model: 6" Still, M/s Pope Scientific Inc., WI 53080, USA) having an evaporating surface of 0.2194 sq.m is pictured in Figure 3.4a. The main unit consisting of evaporating body, jacket for thermic fluid circulation and condenser are made of stainless steel while feed vessel, distillate flask, residue flask and cold trap are made of glass. The schematic diagram of the wiper blade which facilitates the feed down the evaporator body is in Figure 3.4.b.



(a) (b)

Fig. 3.4. (a) Photo of main unit of molecular distillation used in the study. (b) wiper blade details.

The evaporator body is heated by thermostatically controlled 12kW thermic fluid heater (Model: XC2 F12-00, M/S Mokon, Buffalo, NY, USA). The thermic fluid circulates in the jacket of the evaporating body maintaining the desired temperature. The unit is maintained under high vacuum (measured by Pirani gauge) by rotary vane vacuum pump (Model: RV12, M/s Edwards Ltd., England). There are sensors placed at appropriate ports in the unit to measure temperature of feed material, distillate, residue and non-volatile vapors. The cold trap is filled with ice to trap the condensable volatiles that otherwise escape in the vacuum line and damage the vacuum pump. Hot water at 30°C is circulated through the condenser to condense the evaporating molecules.

The extract so obtained by the $SCCO_2$ extraction method outlined in section 3.2.2 is taken to molecular distillation for enrichment of bakuchiol. The extract (100 ml) is admitted into the feed flask of the molecular distillation system, and subjected to the distillation under high vacuum. The feed material is spread into a thin film by a rotating wiper blade assembly driven at a predetermined speed measured in revolutions per minute (RPM) by a DC motor. The film, while being forced into turbulent flow by the wiper blades, flows down the inside surface of the evaporator

body aided by gravity and the slots in the wiper blades. Heated walls and high vacuum drive the more volatile components (distillate) to the closely positioned internal condenser as the less volatile components (residue or raffinate) continue to pour down the evaporator body. The volatiles (distillate) condensed on the condenser surface flows down and collect in the distillate flask. The unevaporated non-volatiles, thus separated, exit through discharge outlets. Each collected fractions are weighed and analyzed for bakuchiol and psoralen contents based on HPLC analysis. Number of experiments are conducted at varying operating conditions temperature, vacuum and wiper speed as per the Box-Behnken design of experiment outlined later in section 3.2.5.

3.2.4. HPLC analysis

See chapter 2.2.5.

3.2.5. Experimental design

When the problem involves the data that are subject to experimental errors, statistical methods measure the effects of change in operating variables and their mutual interactions on the process performance through factorial experimental designs. The experimental design chosen for the study is a factorial Box–Behnken design [Box and Behnken, 1960 and Montgomery, 1991] that helps in investigating linear, quadratic, and cross-product effects of three factors, each varied at three levels. The design involves 15 experiments from three important variables of the system (vacuum, temperature and wiper speed) at three levels (high, medium and low). The design also includes three center points for replication. The actual variables employed and their corresponding coded levels as per the Box-Behnken design are given in Table 3.2.

The relation between the coded and natural variables, for vacuum, evaporation temperature and wiper speed is given as:

$$X_1 = (V_d - V_c) / \Delta V;\ X_2 = (T_d - T_c) / \Delta T;\ X_3 = (S - S_c) / \Delta S \qquad (3.1)$$

Where V_d is the vacuum in molecular distillation unit, μm Hg; T_d is the distillation temperature, °C; S is the wiper speed in RPM. V_c, T_c and S_c are the centre of the range of variables. X_1, X_2 and X_3 are coded values of variables V_d, T_d and S; ΔV, ΔT and ΔS are the increments of V_d, T_d and S corresponding to one unit of X_1, X_2 and X_3.

Table 3.2. Coded and actual values of variables as per Box-Behnken design.

Coded values			Actual values		
X_1	X_2	X_3	V_d	T_d	S
Vacuum	Temperature	Wiper speed	Vacuum, µm Hg	Temperature, °C	Wiper speed, RPM
1	1	0	2000	200	160
1	-1	0	2000	140	160
-1	1	0	400	200	160
-1	-1	0	400	140	160
1	0	1	2000	170	200
1	0	-1	2000	170	120
-1	0	1	400	170	200
-1	0	-1	400	170	120
0	1	1	1200	200	200
0	1	-1	1200	200	120
0	-1	1	1200	140	200
0	-1	-1	1200	140	120
0	0	0	1200	170	160
0	0	0	1200	170	160
0	0	0	1200	170	160

The response function selected to study the effects of the variables on the bakuchiol content is the second order polynomial of the form:

$$Y= A_o + A_1 X_1 + A_2 X_2 + A_3 X_3 + A_{11} X_1^2 + A_{22} X_2^2 + A_{33} X_3^2 + A_{12} X_1 X_2 + A_{13} X_1 X_3 + A_{23} X_2 X_3 + \varepsilon. \quad (3.2)$$

where Y_b is bakuchiol content in the distillate; A_0 is Constant; A_1,A_2,A_3 are linear coefficients; A_{11}, A_{22} ,A_{33} are quadratic coefficients; A_{12} ,A_{13} , A_{23} are cross-product coefficients; ε is the error in the estimate.

The fitness of the model is evaluated by the correlation coefficient (R) and analysis of variance (F-test). The F-test is applied to confirm whether the variance explained by the regression model is significantly larger than the variation of the residual. The coefficients of the response function, their statistical significance and process conditions for maximum bakuchiol content in the distillate were evaluated by the method of least squares by using the software (KyPlot, Version 2.0). Response surfaces based on the predicted polynomial equation along with the contour lines that denote equal bakuchiol contents are also drawn using the KyPlot software. Response surfaces along with contour lines are drawn keeping one variable constant at mid level and varying the other two variables within the range of experiment.

3.3. Results and Discussion:

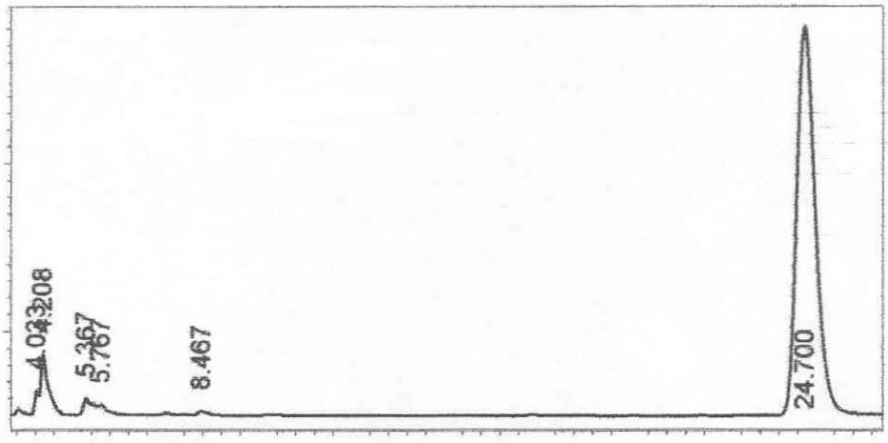
The bakuchiol content in *P. corylifolia* seeds was 9%. The bakuchiol and psoralen content in $SCCO_2$ extracted oil was 29% and 2.9% respectively. The seeds taken for the molecular distillation studies are of different type and the $SCCO_2$ extract contained less bakuchiol and more psoralen content compared to the samples taken for studies in chapter 2. The $SCCO_2$ extract was the feed material to the molecular distillation unit in order to enhance the bakuchiol content in the extract.

Experimental data obtained based on design of experiment showing bakuchiol content in distillate and raffinate at various processing conditions is in Table 3.3. Bakuchiol content in distillate varied from 29.6% to 70.7%. The psoralen content in the distillate was observed to go up to 18.6%. HPLC diagrams of the feed material and each of the distillate obtained at different process conditions indicated peaks pertaining to the key components bakuchiol and psoralen (Fig. 3.5 to Fig. 3.7). The bakuchiol peak was at about 24 min. and psoralen peak was noticed at about 4 min. It was noticed that the psoralen always distills along with the bakuchiol and complete separation of bakuchiol from psoralen is not possible within the range of process conditions employed in this study.

Table 3.3. Experimental and predicted values as per the response surface model

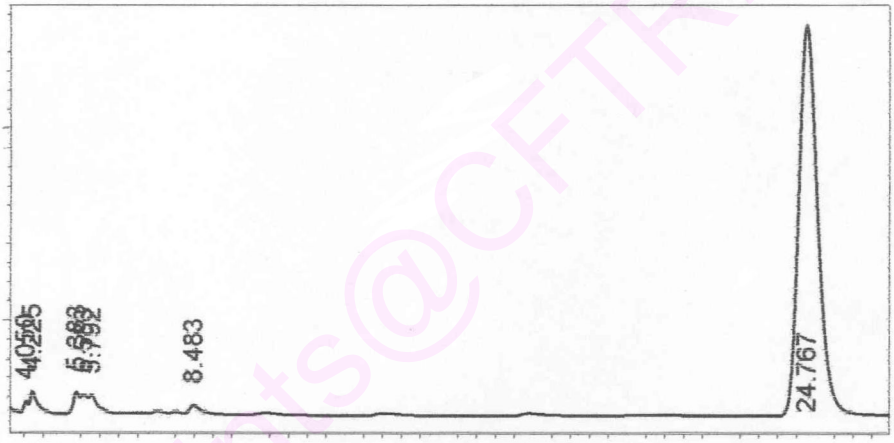
Expt. No.	V_d	T_d	S	Bakuchiol % in distillate		absolute relative deviation, %	Psoralen % in distillate – experimental
				Experimental	Predicted		
1	2000	200	160	67.6	68.6	1.6	9.6
2	2000	140	160	29.6	30.0	1.3	2.8
3	400	200	160	64.0	60.9	4.9	7.5
4	400	140	160	70.7	69.6	1.5	8.2
5	2000	170	200	53.6	48.1	10.2	13.5
6	2000	170	120	39.6	40.9	3.3	16.9
7	400	170	200	54.8	53.5	2.4	18.6
8	400	170	120	62.0	67.5	8.8	8.5
9	1200	200	200	60.9	65.3	7.2	0.9
10	1200	200	120	67.0	64.7	3.5	17.0
11	1200	140	200	44.0	46.3	5.4	14.8
12	1200	140	120	58.1	53.7	7.6	16.4
13	1200	170	160	53.9	57.2	6.1	13.1
14	1200	170	160	48.0	57.2	19.3	9.8
15	1200	170	160	69.8	57.2	18.0	12.4
16*	2000	200	200	70.8	72.0	1.7	12.9

* Experiment conducted at predicted optimum process conditions



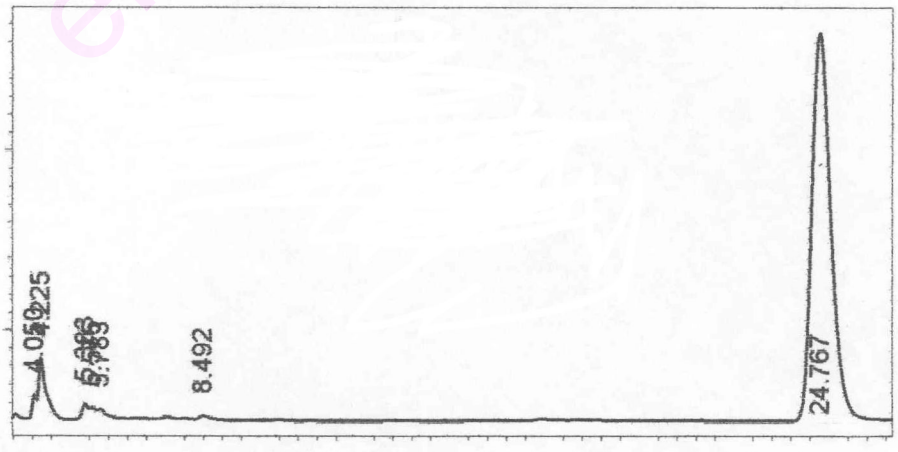
Time, min.

Fig. 3.5. HPLC chromatogram of $SCCO_2$ extract that is feed material to molecular distillation.



Time, min.

Fig. 3.6. HPLC chromatogram of raffinate of molecular distillation at vacuum of 2000 µm, 200 °C and 160 rpm.



Time, min.

Fig. 3.7. HPLC chromatogram of distillate of molecular distillation at vacuum of 2000 µm, 140 °C and 160 rpm.

The experimental values of bakuchiol content in the distillate regressed well when the data were fitted to second order polynomial equation as seen from high correlation coefficient (Table 3.4). The coefficients of the response model along with statistical analysis were listed in Table 3.4.

Table 3.4. Regression analysis for the response surface equation fitting the experimental data along with Coefficients of the response surface equation.

Regression Statistics:

Correlation coefficient, R	0.904
Standard Error	8.822

ANOVA:

	degrees of freedom	sum of squares	mean sum of squares	F ratio	Significance F
Regression	9	1740.9	193.4	2.49	0.16
Residual	5	389.2	77.8		
Total	14	2130.1			

Coefficients	Value	Standard Error	t Stat	P-value
A_o	251.23	201.42	1.25	0.27
A_1	-0.111	0.04	-2.64	0.05
A_2	-1.571	1.85	-0.85	0.43
A_3	-0.072	1.13	-0.06	0.95
A_{11}	-3.86E-06	0.00	-0.54	0.61
A_{22}	2.83E-03	0.01	0.55	0.60
A_{33}	-1.41E-03	0.00	-0.49	0.64
A_{12}	4.93E-04	0.00	2.68	0.04
A_{13}	1.65E-04	0.00	1.20	0.28
A_{23}	1.67E-03	0.0037	0.45	0.67

ANOVA analysis indicated the high significance of the linear term of vacuum and cross-product term of vacuum-temperature as evidenced by very low P values ($P \leq 0.05$).

The effect of process variables on the bakuchiol content in distillate was studied based on response surface curves (Figures 3.8, 3.9 and 3.10). Figure 3.8 illustrates that bakuchiol content increased with respect to increase in both vacuum and temperatures at a constant wiper speed of 160 RPM. There is a region of operating conditions (between temp. 158 to 200°C and vacuum levels of 400-800 μm Hg) in which it is not possible to obtain higher bakuchiol content in the distillate.

Figure 3.9 shows the predicted effect of vacuum and wiper speed on bakuchiol content in distillate at temperature of 170°C. Lower wiper speed and higher vacuum levels favored the increased bakuchiol content in distillate. Figure 3.10 shows the response surface along with contour lines for the variation in wiper speed and temperature at a constant vacuum level of 1200 μm.

The response surface equation was searched for maximum bakuchiol content in distillate within the range of process conditions. Maximum bakuchiol content was predicted to occur at vacuum level of 2000 μm, temperature of 200°C and wiper speed of 200 RPM. Experiment conducted at the above conditions resulted in 70.8% bakuchiol content in the distillate showing good agreement with the response surface model (predicted bakuchiol content was 72%). The results indicated that response surface model methodology can be successfully employed to predict the effect of process conditions on the bakuchiol yield and one can also obtain maximum enrichment of bakuchiol content in distillate byoptimizing the response equation.

There were only few reports on application of molecular distillation on bioactive materials out of which one study pertained to optimizing the process parameters of molecular distillation by adopting response surface methodology. The study pertains to recovery of tocopherol from rapeseed deodorizer distillate. Optimum temperature, feed rate and wiper speed were obtained based on response surface model. The model was found to describe the process adequately [Jiang *et al*., 2006]. Another study utilized RSM technique to optimize the tocopherol recovery that dealt with deacidification of grape seed oil using molecular distillation. The influence of two operating variables feed flow and evaporation on tocopherol recovery was

studied. It was recommended that high flow rate and high temperature help in tocopherol recovery of 100% while keeping free fatty acid content in the product low [Martinello *et al*., 2006].

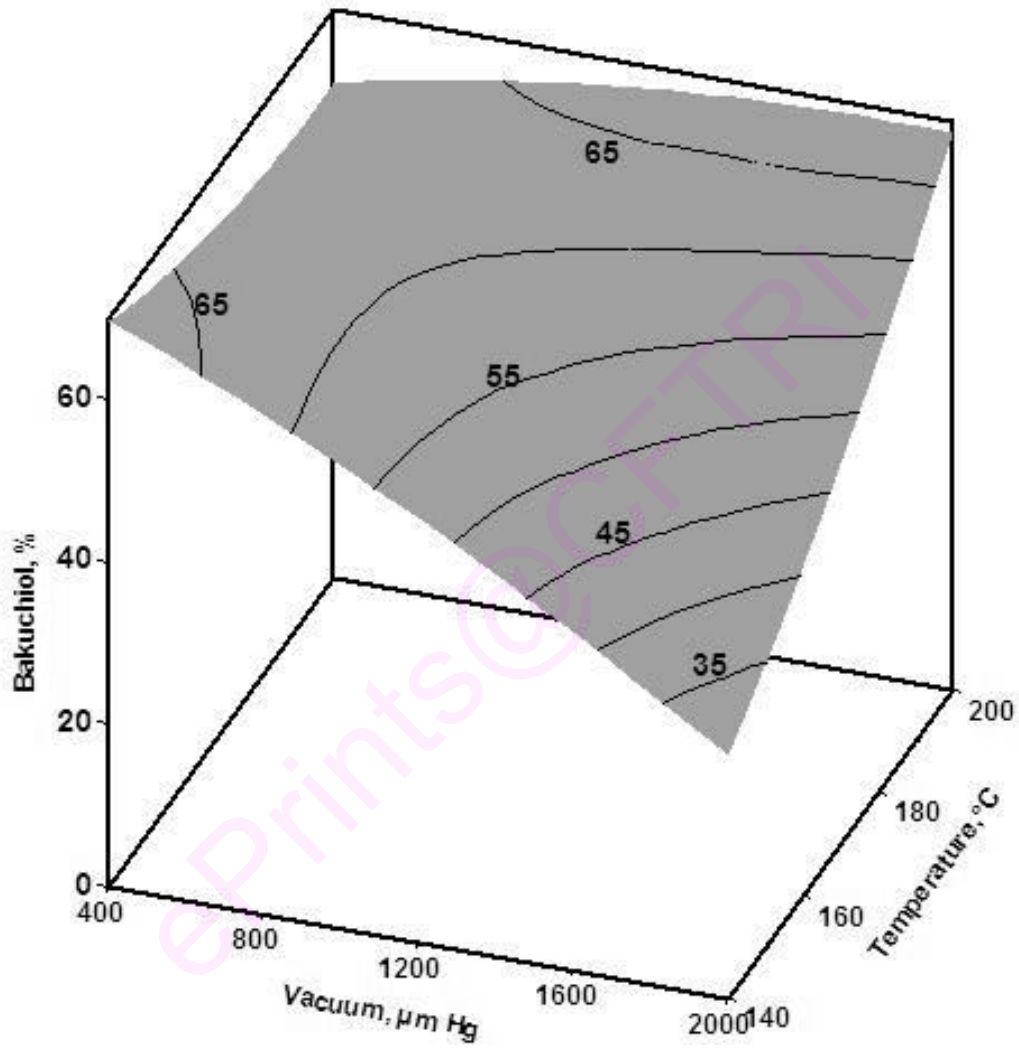


Fig. 3.8. Surface and contour plot diagram showing the predicted effect of vacuum and temperature on bakuchiol content. Wiper speed is fixed at 160 RPM.

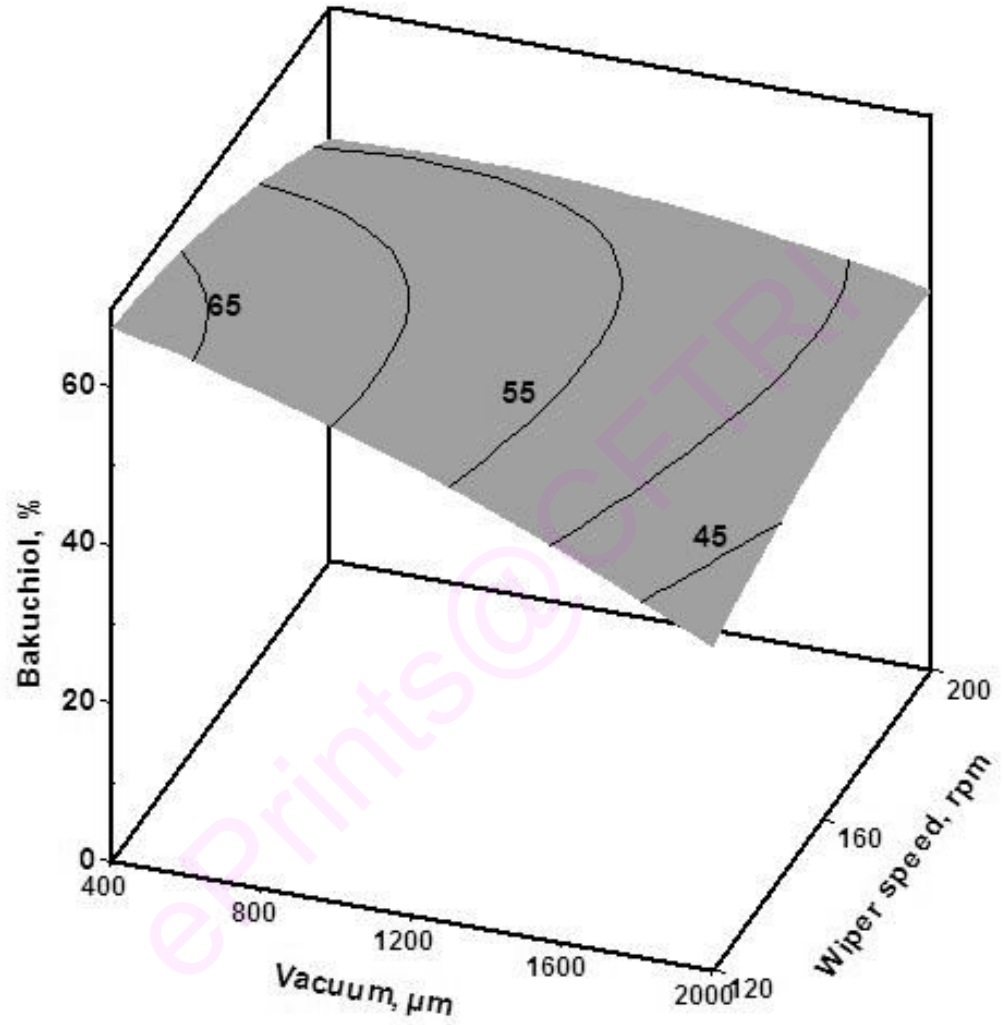


Fig. 3.9. Surface and contour plot diagram showing the predicted effect of vacuum and wiper speed on bakuchiol content. Temperature is fixed at 170°C.

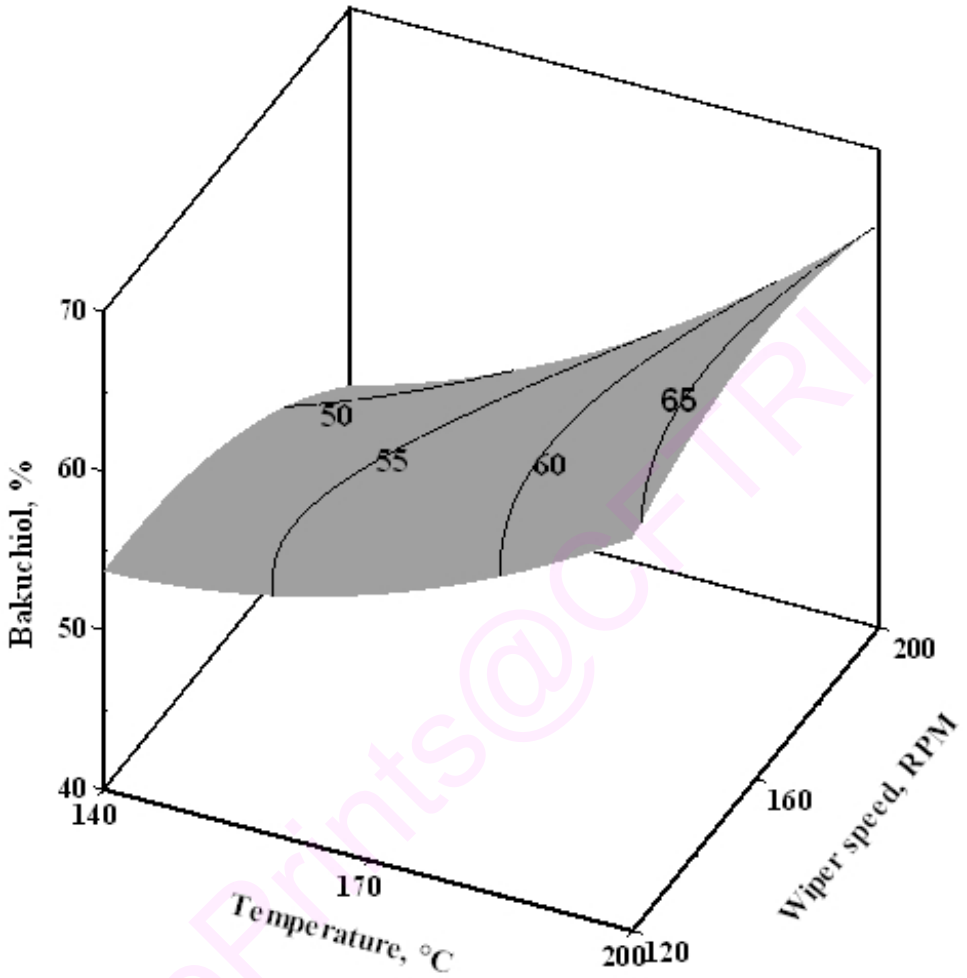


Fig. 3.10. Surface and contour plot showing the predicted effect of temperature and wiper speed on bakuchiol content. Vacuum is fixed at 1200 µm Hg.

Psoralen content in SCF extracted oil was 2.9%. Psoralen co-distills with bakuchiol and complete removal of psoralen was not possible under the process conditions employed. Under the optimum process conditions indicated by the response surface model, the distillate contained psoralen of 12.9% whereas the bakuchiol content was 70.8%.

3.4. Conclusion

Psoralea corylifolia seed extract was subjected to supercritical CO_2 extraction followed by molecular distillation with the objective of enhancing the bakuchiol content in the extract. Effect of process variables such as pressure (400-2000 μm), temperature 140–200 °C) and wiper speed (120–200 RPM) was studied based on Response Surface Methodology. Experimental data regressed well with second order polynomial model with interaction terms. The predicted model was maximized for bakuchiol content in the distillate. A maximum of 72% bakuchiol in distillate was predicted to be possible under the optimized process conditions and the experiment at the optimum conditions yielded distillate with 70.8% bakuchiol. Further, it was observed that both principal components bakuchiol and psoralen co-distill during the molecular distillation.

3.5. Nomenclature

A	area
A_0	Constant in equation 3.2
A_1,A_2,A_3	Linear coefficients in equation 3.2
A_{11}, A_{22} ,A_{33}	Quadratic coefficients in equation 3.2
A_{12} ,A_{13} , A_{23}	Cross-product coefficients in equation 3.2
M_w	Molecular weight of the compound
N	Number of moles of vapor condensing on the surface
P_g	Vapor pressure of pure material
R	Correlation coefficient
R_g	Universal gas constant
S	Wiper speed
T_d	Distillation temperature
T	Absolute temperature
V_c, T_c , S_c	centre of the range of variables V_d, T_d and S
V_d	Vacuum in molecular distillation unit
X_1, X_2 , X_3	coded values of variables V_d, T_d and S respectively
Y_b	bakuchiol content in the distillate
ΔV, ΔT, ΔS	increments of V_d, T_d and S corresponding to one unit of X_1, X_2 and X_3
ε	error in the estimate
τ	time

CHAPTER 4

Enzymatic synthesis of bakuchiol glycosides

4.0. Introduction

In view of the conventional notion that enzymes are active only in water, enzymatic catalysis is normally carried out primarily in aqueous systems. Although water is a poor solvent for preparative organic chemistry, it is the unique specificity of enzymes that drew the interest of chemists who were seeking highly selective catalytic agents. Enzymatic reactions in nonaqueous solvents offer numerous possibilities for the biotechnological production of useful chemicals using reactions that are not feasible in aqueous media. Non-aqueous media for enzymatic catalysis may be categorized as: water: water-miscible (monophasic aqueous-organic system); water: water-immiscible (biphasic aqueous-organic system); nonaqueous (monophasic organic system); anhydrous; supercritical fluids; reversed micelles; solvent-free systems; gas phase; and ionic liquids [Hari Krishna, 2002]. These reactions include chiral synthesis or resolution; production of high-value pharmaceutical substances; modification of fats and oils; synthesis of flavor esters and food additives; production of biodegradable polymers, peptides, proteins, and sugar-based polymers.

In nonaqueous solvents, hydrolytic enzymes could undergo synthetic reactions while they also exhibit altered selectivities, pH memory, increased activity and stability at elevated temperatures, regio-, enantio- and stereoselectivity, and may also be affected by their water activity. Currently, there is a considerable interest in the use of enzymes (particularly lipases, esterases, and proteinases) as catalysts in organic synthesis [Divakar and Manohar, 2007]. In a recent review on supercritical fluids as solvents for enzymatic reactions, different types of enzymatic reactions that have been carried out in non-aqeous media particularly, $SCCO_2$, SC-SF_6, n-hexane, tolune, ionic liquids, biphasic KKP-buffer, di-isoprophyl ether, and ethanol have been tabulated elaborately [Habulin *et al*., 2007].

One research area which is to yet to be fully exploited is the use of supercritical fluid as a reaction medium in which the fluid either actively participates in the reaction or functions solely as the solvent medium for the reactants, catalysts or products. In one of the first reactions in $SCCO_2$, the hydrolysis of disodium salt of p-nitrophenyl phosphoric acid reacting to p-nitrophenol has been reported [Randolph *et*

al., 1985]. Similar reaction has also been demonstrated for polyphenyl oxidase [Hammond *et al*., 1985]. In general, replacement of conventional liquid solvents by SCFs can increase the rate and tune the selectivity of reactions for the following reasons [Knez *et al*., 2007]:

1. Rapid diffusion of solutes or weakening of solvation around reacting species facilitating the reactions and changing the reaction pathways occasionally.
2. Local clustering of solutes or solvents resulting in an appreciable increase in the local concentration of substrate (and catalyst) causing acceleration of the reaction.
3. Reduction or increase in the cage effect often affects the reaction performance of rapid chemical transformations such as radical reactions.

In addition to the above benefits, $SCCO_2$, as a reaction media, results in economical, technical, environmental and health advantages. The high volatility of CO_2 allows it to be completely and easily removed from the product, resulting in an overall "solvent-free" reaction. By using $SCCO_2$, an integrated production process can be performed, because it can act as solvent for the reaction and also as a separation medium. The variable solvating power (which depends on pressure and temperature) of $SCCO_2$ facilitates the integration of biocatalytic and downstream processing steps in a single robust bioreactor.

Benefits of using SCFs for improved reaction rates, control of selectivities by pressure, and so forth in enzymatic reactions have been well demonstrated [Mori and Okahata, 1998, 2000, 2002; Kamat *et al*., 1993 and Chaudhary *et al*.,1996]. For example, since the first reports on the use of SCF as reaction media, studies such as enzymatic oxidation [Knez *et al*., 1998], hydrolysis [Turner *et al*., 2001; Nagesha *et al*., 2006], transesterification [Yu *et al*., 1999], esterification [Chi *et al*., 1988 and 2006], interesterification [Nelson, 1996] and enantioselective synthesis [Miller *et al*., 1990; Compton and King, 2001] in SCF have strengthened the feasibility of enzymatic reactions in supercritical fluids. Frequently, the temperature range employed in supercritical carbon dioxide processing is compatible with the use of enzymes as catalysts in view of the lower critical temperature (31.1°C) of CO_2. An additional benefit of using supercritical fluids along with enzymatic catalysis is that it provides a medium for the recovery of products or reactants without many unwanted steps.

4.1. Glycosides

Glycosides are asymmetric mixed acetals formed by reaction 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. Glycosides are widely distributed in nature and can be found in nearly every living organism [Roode *et al.,* 2003]. An overview about synthesis and characterization of glycosides has been covered [Marco, 2007].

Glycosides are readily soluble in water and crystallize easily. Their aqueous solutions, if not attacked by microorganisms, are stable, do not exhibit mutarotation and are non-reducing. 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 applications in industries and pharmaceuticals [Stanek *et al.,* 1963].

In contrast to free carbohydrate, the glycosides are not sweet (exceptions such as methyl α-D-glucopyranoside). They taste more or less bitter. 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. Unsubstituted glycosides are less sensitive to oxidative agents than free carbohydrate. 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.

Glycosides are useful in 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 flavor and fragrances are used in perfumery [Wantanabe *et al.,* 1993; 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. Syntheses of vanillyl maltoside by glucosidase [Sivakumar

et al., 2007] and kinetic studies on synthesis of vanillin and curcumin [Sivakumar *et al.*, 2006] have been reported.

4.1.1. Enzymatic glycosylation

Enzymatic glycosylation includes the use of glycosides under reverse hydrolytic conditions. There are many advantages of using glycosidases [Takayama *et al*., 1997] and some of them are: high regio and stereoselectivity; mild reaction conditions and biocompatibility; replace wasteful or expensive chemical procedure with more efficient semi-natural processes; improved product-yield and better product quality; use of nonpolar solvents which imparts stability to glycosidases renders insolubility of the enzyme, solubility of alcohols and products in organic solvents and easy product workout procedures; no protection, activation and deprotection required and less environmental pollution. Table 4.1 lists some of the important phenolic, sweetener and medicinal glycosides cited in the literature along with source of enzymes and field of application of such glycosides.

4.1.2. Glycosylation in supercritical carbon dioxide

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 [DeSimone, 2002; Beckmann, 2003; Rezaci *et al*. 2007].

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; Oakes *et al.,* 2001; Mori and Okahata, 2002; Matsuda *et al.,* 2002].

Table 4.1. Some selected glycosides prepared through enzymatic glycosylation

Name of the compound	Source of enzyme	Applications	References
A. Phenolic glycosides			
Vanillin-β-D-monoglucopyranoside	Cell suspension culture of *Coffea arabica*	As a food additive flavor	Kometani *et al.* 1993b, 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.* 1993a Hamada *et al.* 2003
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, cistanosideA,cistanoside B	Plant cell culture of *Cistanche deserticola*	Antinociception, anti-inflammatory, sedation	Ouyang *et al*. 2005

Name of the compound	Source of enzyme	Applications	References
B. Sweetener glycosides			
Stevioside	Cell free extract of *Gibberella fujikuroi*	Natural sweetener	Oliveira *et al*. 2007; Brandle and Telmer 2007
Stevioside, steviobioside, rebaudioside A, rebaudioside B	Leaves of *Stevia rebaudiana*	As natural sweeteners, utilized in beverages	Kohda *et al.* 1976
Steviol-13-*O*-glucopyranoside, stevio-bioside, stevioside and rebaudioside	Soluble extracts of stevia	As natural food sweeteners	Shibata *et al.* 1991
C. Glycosides in medicine			
Enedyne antibiotics-calicheamycin	Cultivation broth of *Micromonospora echinospora*	Antitumor agents	Lee *et al.* 1987; Golik *et al.* 1987
Esperamycins	*Actinomadura verrucosospora*	Antitumor activity	Long *et al.* 1989
D. Steroidal glycosides			
Eryloside A, eryloside K, eryloside L	Marine sponge of *Erylus lendenfeldi*	Anticancer, antifungal, anticoagulative agent,	Sandler *et al*. 2005
Glycosides of diosgenin, solasodine, solasonine	*Solanum* sp.	Anticarcinogenic activity	Nakamura *et al.* 1996

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 Chang, 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].

Enzymatic glycosylation of bakuchiol with different carbohydrates in supercritical fluid media has not been reported in literature. Hence, the present study is attempted to prepare water soluble bakuchiol glycosides enzymatically using amyloglucosidase from *Rhizopus* mold utilizing different sugars in two different environments: one under conventional reflux conditions and another under $SCCO_2$ conditions.

4. 2. Materials and Methods

4.2.1. Enzymes

Amyloglucosidase from *Rhizopus* sp. is purchased from Sigma Company, St. Louis, MO, USA. Amyloglucosidase activity [Sumner and Sisler, 1944] is found to be 11.2 activity units (AU-μmol/(mg. enzyme. min)).

4.2.2. Chemicals and reagents

D-Galactose and D-fructose are procured from HiMedia Pvt. Ltd, India. D-Glucose and sucrose purchased from SD Fine Chemicals (India) Ltd., D-mannose, D-arabinose, D-ribose, D-sorbitol and D-mannitol, from Loba Chemie Pvt. Ltd., India and maltose from Sigma Chemical Co., St. Louis, MO, USA are employed. Lactose, HPLC grade acetonitrile and di-isopropyl ether are from Sisco Research Laboratories Pvt. Ltd. India. Di-isopropyl ether is distilled once before use.

4.2.3. Glycosylation procedure - conventional reflux method

Syntheses of bakuchiol glycosides involves refluxing bakuchiol (0.5 mmol) with 1.0 mmol carbohydrates in 100 ml di-isopropyl ether in presence of amyloglucosidase (40% w/w carbohydrates), Dimethyl formamide (DMF) 5.0 ml and 0.1mM (in 100 ml di-isopropyl ether), pH 6.0 buffer for an incubation period of 72 h at 68 °C (Scheme 4.1). Phosphate buffer of pH 6.0 is employed in the reaction. After the reaction, the solvent is evaporated and the enzyme denatured at 100 °C by holding in a boiling water bath for 5-10 min. The residue containing unreacted bakuchiol, carbohydrates, along with the product glycosides are dissolved in 15-20 mL of water and the reaction mixture extracted with hexane to remove unreacted bakuchiol. The dried residue is subjected to HPLC analysis to determine the extent of conversion. All the reactions are performed in duplicate and the mean values are shown in tables. Unreacted carbohydrate is separated from the product glycosides by size exclusion chromatography using Sephadex G15 column (100 cm x 1 cm), eluting with water at 1mL/h rate. Individual glycosides could not be separated satisfactorily, due to similar polarity of the glycosides formed.

Syntheses of the other bakuchiol glycosides are carried out at the above determined conditions, with bakuchiol and carbohydrates: aldohexoses – D-glucose, D-galactose and D-mannose; ketohexose – D-fructose; pentoses – D-ribose and D-arabinose; disaccharides – maltose, lactose and sucrose; sugar alcohol – D-sorbitol and D-mannitol. The conditions employed with the enzymes are: bakuchiol (0.5 mmol) and carbohydrate (1.0 mmol), amyloglucosidase (40% w/w carbohydrates), 0.2 mM, pH 6.0 phosphate buffer and 72h of incubation period.

4.2.4. Glycosylation procedure - under $SCCO_2$ condition

Syntheses of the bakuchiol glycosides are carried out with bakuchiol and carbohydrates under supercritical CO_2 atmosphere: 100 bar pressure at 50°C. The reactor vessel along with the CO_2 supply system is shown schematically in Figure 4.2.

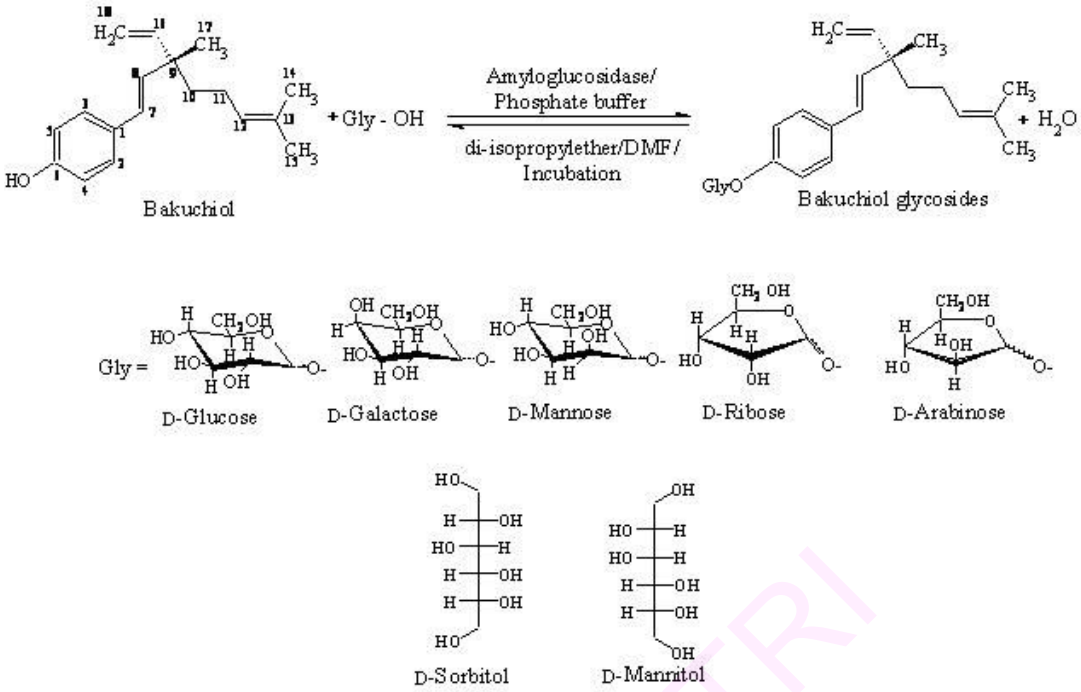


Fig. 4.1. Scheme for synthesis of bakuchiol glycosides

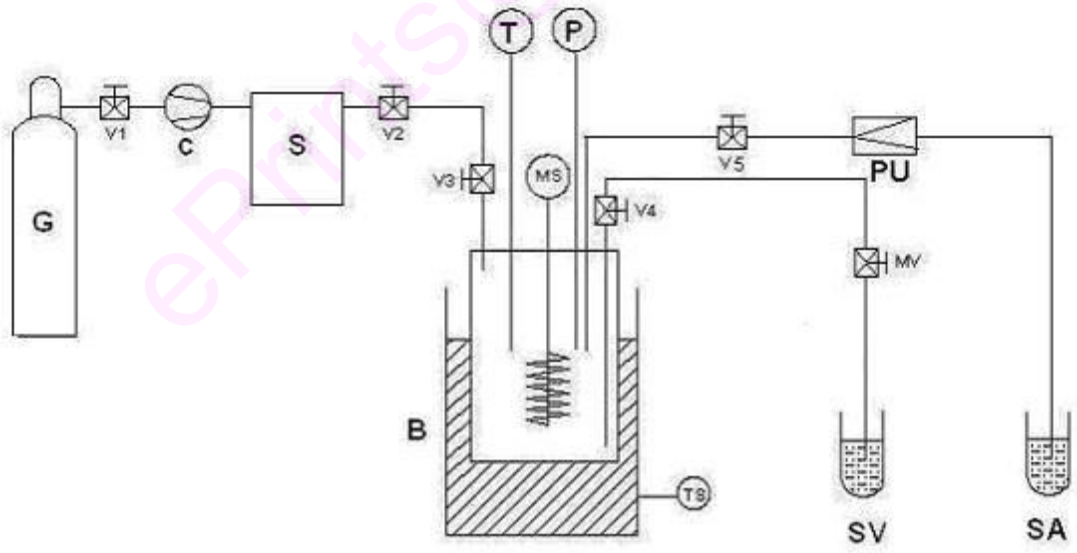


Fig. 4.2. Reaction setup for glycosylation in supercritical CO_2 media.

G, Gas cylinder; C, Compressor; S, Surge tank; B, Berghof autoclave; T, Temperature indicator; P, Pressure indicator; MS Magnetic stirrer; V1-V5, High pressure needle valve; MV, micrometer valve; SV, Sampling valve; SA, Saline solution; TS thermostat; PU, pump.

The system consists of a reactor of 120 ml capacity with a magnetic stirrer and a recirculating fluid loop by means of a pressure differential for sampling through a Rheodyne valve with 0.5 ml loop for sampling. Total volume of about 50 ml of the reactor vessel is thermostatically controlled to maintain a constant temperature. Process conditions employed for the reaction are: bakuchiol (0.5 mmol) and carbohydrate (1.0 mmol), amyloglucosidase (40% w/w carbohydrates), DMF 15 ml, and 0.1 mM, pH 6.0 phosphate buffer and 24 h of incubation period. The CO_2 is then released and the reaction products are taken out in 15-20 ml of water, evaporated to dryness and subjected to analyses by HPLC and NMR.

4.2.5. Antioxidant activity measurement

Antioxidant activity of bakuchiol glycosides are determined by DPPH (2,2 diphenyl-1-picryl hydrazyl) radical scavenging method [Moon and Tearo, 1998]. The procedure is outlined in Chapter 2.2.8.

4.2.6. Angiotensin converting enzyme (ACE) inhibition assay

ACE inhibition assay for the bakuchiol glycosides are performed on to ACE isolated from pig lung by the Cushman and Cheung method [Cushman and Cheung, 1971]. The procedure is outlined in Chapter 2.2.9.

4.2.7. ^{1}H and ^{13}C nuclear magnetic resonance

Two dimensional Heteronuclear Single Quantum Coherence Transfer spectra (2D HSQCT) are recorded on a Brüker DRX-500 MHz spectrometer operating at 500.13 MHz for ^{1}H and 125 MHz for ^{13}C at 35 °C. Proton and carbon 90° pulse widths are 12.25 and 10.5 μs, respectively. Chemical shifts are expressed in ppm relative to internal tetramethylsilane standard. About 40mg of the glycoside sample dissolved in DMSO-d_6 is 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 100 μs to defocus unwanted coherences. Increment of t_1 is in 256 steps with a computer memory size of 4kB. The spectra are processed using unshifted and $\pi/4$ shifted sine bell window function in F_1 and F_2 dimensions, respectively.

4.2.8. Product characterization

Isolated glycosides besides measuring melting point and optical rotation are also characterized by recording UV, IR, Mass and 2D-HSQCT NMR spectra which provided good information about the nature and type of products. In 2D-HSQCT some of the assignments are interchangeable. Only resolvable signals are shown. The 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 signals.

4.2.9. Spectral characterization

Bakuchiol: Solid, UV (λ_{max}): 226.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{226.5}$ – 3481 M^{-1}), 299.0 nm ($n\rightarrow\pi^*$, $\varepsilon_{295.5}$ – 896 M^{-1}); IR (KBr, stretching frequency, cm^{-1}): 3320 (OH), 1373 (C=C), 2928 (CH); 2D-HSQCT (DMSO-d_6) ^{1}H NMR δ_{ppm} (500.13): 7.27 (H-2), 7.19 (H-3), 6.69 (H-4), 6.71 (H-5), 6.04 (H-7), 6.17 (H-8), 1043 (H-10a), 1.21 (H-10b), 1.95 (H-11), 4.66 (H-12), 1.62 (H-14), 1.52 (H-15), 5.9 (H-16), 1.14 (H-17), 5.08 (H-18a), 5.02 (H-18b); ^{13}C NMR δ_{ppm} (125 MHz): 126.6 (C1), 127.2 (C2), 127.2 (C3), 115.4 (C4), 115.4 (C5), 156.1 (C6), 128.4 (C7), 134.1 (C8), 42.2 (C9), 41.0 (C10), 25.5 (C11), 124.2 (C12), 130.6 (C13), 23.1 (C14), 22.9 (C15), 146.0 (C16), 17.5 (C17), 111.8 (C18).

6-*O*-(D-Glucopyranosyl)bakuchiol: Solid, UV (λ_{max}): 191.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{191.5}$ – 3074 M^{-1}), 229.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{229.5}$ – 1774 M^{-1}), 275.5 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{275.5}$ – 511 M^{-1}); IR (KBr, Stretching frequency, cm^{-1}): 3371 (OH), 1381 (glycosidic aryl alkyl C-O-C asymmetrical), 1080 (glycosidic aryl alkyl C-O-C symmetrical), 1380 (C=C), 2937 (CH); MS (m/Z) 419$[M+1]^+$. 2D-HSQCT (DMSO-d_6) **C1α-glucoside**: ^{1}H NMR δ_{ppm} (500.13) **Glu**: 4.77(H-1α, d, J = 2.7 Hz), 3.57 (H-3α), 3.11 (H-4α); **Bakuchiol**: 7.26(H-3), 2.01(H-11), 4.9(H-12), 0.93(H-17); ^{13}C NMR δ_{ppm} (125 MHz): **Glu**: 95.0 (C1α), 75.5 (C3α), 70.8 (C4α), 63.3 (C6α); **Bakuchiol**: 127.9 (C1), 116.1 (C4), 114.9 (C5), 162.5 (C6), 129.8 (C7); **C1β-glucoside**: ^{1}H NMR **Glu**: 4.27 (H-1β, d, J = 6.7 Hz), 3.67 (H-6a), ^{13}C NMR δ_{ppm} **Glu**: 101.8 (C1β), 75.5 (C2β), 76.3 (C3β).

6-*O*-(D-Galactopyranosyl)bakuchiol: Solid, UV (λ_{max}): 191.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{191.5}$ – 2983 M^{-1}), 231.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{231.5}$ – 1123 M^{-1}), 274.5 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{274.5}$ – 257 M^{-1}); IR (KBr, stretching frequency, cm^{-1}): 3319 (OH), 1246 (glycosidic aryl alkyl C-O-C

asymmetrical), 1064 (glycosidic aryl alkyl C-O-C symmetrical), 1361(C=C), 2917.6 (CH); MS (m/Z) 419[M+1]$^{+}$. 2D-HSQCT (DMSO-d_6) **C1α-galactoside**: ^{1}H NMR δ_{ppm} (500.13) **Gal**: 4.28 (H-1α, d, J = 2.7 Hz), 3.67 (H-2α), 3.76 (H-3α), 3.78 (H-4α), 3.66 (H-5α); **Bakuchiol**: 7.14(H-2), 7.16(H-3), 6.50 (H-4), 6.52 (H-5), 6.13 (H-8), 1.14 (H-10), 1.13 (H-17); ^{13}C NMR δ_{ppm} (125 MHz): **Gal**: 95.4 (C1α), 68.4 (C2α), 74.7 (C4α), 62.7 (C6α); **Bakuchiol**: 130.6 (C3), 162.5 (C6), 15.6 (C17); **C1β-galactoside**: ^{1}H NMR **Gal**: 4.63 (H-1α, d, J = 3.4 Hz), 3.29 (H-1β, d, J = 7.2 Hz), 3.33 (H-5β); ^{13}C NMR δ_{ppm} **Gal**: 101.8 (C1β), 70.7 (C3β), 77.5 (C5β).

6-*O*-(D-Mannopyranosyl)bakuchiol: Solid, UV (λ_{max}): 191.5 nm (σ→σ*, $\varepsilon_{191.5}$ – 2353 M^{-1}), 225.5 nm (σ→σ*, $\varepsilon_{225.5}$ – 120 M^{-1}), 275.0 nm (π→π*, $\varepsilon_{275.0}$ – 47 M^{-1}); IR (KBr, stretching frequency, cm^{-1}): 2925 (OH), 1241 (glycosidic aryl alkyl C-O-C asymmetrical), 1059 (glycosidic aryl alkyl C-O-C symmetrical), 1440 (C=C), 2925 (CH); MS (m/Z) 419[M+1]$^{+}$. 2D-HSQCT (DMSO-d_6) **C1α-Mannoside**: ^{1}H NMR δ_{ppm} (500.13) **Man**: 4.95 (H-1α, d, J = 1.6 Hz), 3.29 (H-3α), 3.02 (H-5α), 3.68 (H-6α); **Bakuchiol**: 1.46 (H-10a), 1.47 (H-10b), 0.922 (H-17); ^{13}C NMR δ_{ppm} (125 MHz): **Man**: 94.76 (C1α); **Bakuchiol**: 132.1(C13), 14.3 (C17).

6-*O*-(D-Fructofuranosyl)bakuchiol: Solid, UV (λ_{max}): 191.5 nm (σ→σ*, $\varepsilon_{191.5}$ – 2064 M^{-1}), 229.5 nm (σ→σ*, $\varepsilon_{229.5}$ – 603 M^{-1}), (n→π*, $\varepsilon_{256.5}$ – 239 M^{-1}), 288.5 nm (π→π*, $\varepsilon_{288.5}$ – 222 M^{-1}); IR (KBr, stretching frequency, cm^{-1}): 3382.8 (OH), 1244 (glycosidic aryl alkyl C-O-C asymmetrical), 1060 (glycosidic aryl alkyl C-O-C symmetrical), 1416 (C=C), 2930.1 (CH); MS (m/Z) 419[M+1]$^{+}$. 2D-HSQCT (DMSO-d_6) **C6-fructoside**: ^{1}H NMR δ_{ppm} (500.13) **Fru**: 3.53 (H-3), 3.69 (H-4), 3.57 (H-5), 3.49 (H-6); **Bakuchiol**: 1.42(H-10a), 1.43 (H-10b), 1.13 (H-17); ^{13}C NMR δ_{ppm} (125 MHz): **Fru**: 104.2 (C2), 70.7 (C3), 72.6 (C4), 71.6 (C5), 63.4 (C6); **Bakuchiol**: 35.9 (C9), 38.2 (C10), 27.1 (C11), 14.0 (C17).

6-*O*-(D-Ribofuranosyl)bakuchiol: Solid, UV (λ_{max}): 191.5 nm (σ→σ*, $\varepsilon_{191.5}$ – 5015 M^{-1}), 222.5 nm (σ→σ*, $\varepsilon_{222.5}$ – 2146 M^{-1}), 260.4 nm (π→π*, $\varepsilon_{260.4}$ – 971 M^{-1}); IR (KBr, stretching frequency, cm^{-1}): 3350 (OH), 1241 (glycosidic aryl alkyl C-O-C asymmetrical), 1085 (glycosidic aryl alkyl C-O-C symmetrical), 1416 (C=C), 2930 (CH); MS (m/Z) 388[M]$^{+}$. 2D-HSQCT (DMSO-d_6) **C1α-riboside**: ^{1}H NMR δ_{ppm} (500.13) **Rib**: 4.64 (H-1α, d, J = 3.6 Hz), 3.78 (H-4α); **Bakuchiol**: 7.25 (H-2), 7.1 (H-

3), 4.95 (H-12); ^{13}C NMR δ_{ppm} (125 MHz) **Rib**: 96.5 (C1α), 71.1 (C2α); **Bakuchiol**: 127.8 (C2), 130.1 (C7), 26.9 (C11); **C1β-riboside**: ^{1}H NMR δ_{ppm} **Rib**: 4.9 (H-1β, d, J = 7.6 Hz), 3.62 (H-4β); ^{13}C NMR δ_{ppm} **Rib**:101.6 (C1β), 70.9 (C3β): **5-*O*-arylated**: ^{1}H NMR **Rib**: 3.55 (H-1α, d, J = 2.9 Hz); ^{13}C NMR δ_{ppm} **Rib**: 62.1 (C5α).

6-*O*-(D-Arabinofuranosyl)bakuchiol: Solid, UV (λ_{max}): 191.5 nm (σ→σ*, $\varepsilon_{191.5}$ – 6351 M^{-1}), 221.4 nm (σ→σ*, $\varepsilon_{221.4}$ – 2701 M^{-1}), 259.5 nm (π→π*, $\varepsilon_{259.5}$ – 1241 M^{-1}); IR (KBr, stretching frequency, cm^{-1}): 3302 (OH), 1240 (glycosidic aryl alkyl C-O-C asymmetrical), 1085 (glycosidic aryl alkyl C-O-C symmetrical), 1404 (C=C), 2926 (CH); MS (m/Z) 427 $[M+K]^{+}$. 2D-HSQCT (DMSO-d_6) **C1α-arabinoside**: ^{1}H NMR δ_{ppm} (500.13) **Ara**: 5.00 (H-1α, d, J = 3.4 Hz), 3.70 (H-4α), 3.52 (H-2α); **Bakuchiol**: 6.34 (H-8), 0.94 (H-17), 5.09 (H-18a), 5.32 (H-18b); ^{13}C NMR δ_{ppm} (125 MHz): **Ara**: 95.9 (C1α), 75.5 (C2α); **Bakuchiol**: 116.1 (C4), 127.90 (C2), 28.68 (C11); **C1β-arabinoside**: ^{1}H NMR δ_{ppm} **Ara**: 4.96 (H-1β, d, J = 6.2 Hz), 3.40 (H-4β); ^{13}C NMR δ_{ppm} **Ara**:102.1 (C1β), 77.4 (C2), 65.0 (C5β).

6-*O*-(1-D-Sorbitol)bakuchiol: Solid, UV (λ_{max}): 191.0 nm (σ→σ*, $\varepsilon_{191.0}$ – 2092 M^{-1}), 226.0 nm (σ→σ*, $\varepsilon_{226.0}$– 446 M^{-1}), 255.0 nm (π→π*, $\varepsilon_{255.0}$ – 242 M^{-1}); IR (KBr, stretching frequency cm^{-1}): 3384 (OH), 1257 (glycosidic aryl alkyl C-O-C asymmetrical), 1062 (glycosidic aryl alkyl C-O-C symmetrical), 1365 (C=C), 2923 (CH); MS (m/Z) 419$[M+1]^{+}$. 2D-HSQCT (DMSO-d_6) **C1-sorbitol**: ^{1}H NMR δ_{ppm} (50013) **Sor**: 3.26(H-1); **Bakuchiol**: 1.87 (H-11), 1.48 (H-14); ^{13}C NMR δ_{ppm} (125 MHz): **Sor**: 60.5 (C1), 74.4 (C2), 71.0 (C3), 73.3 (C4), 72.4 (C5); **Bakuchiol**: 26.7 (C11), 22.2 (C15), 14.0 (C17).

6-*O*-(6-D-Mannitol)bakuchiol: Solid, UV (λ_{max}): 191.5 nm (σ→σ*, $\varepsilon_{191.5}$ – 9000 M^{-1}), 199.5 nm (σ→σ*, $\varepsilon_{199.5}$ – 6557 M^{-1}), 209.0 nm (σ→σ*, $\varepsilon_{209.0}$ – 6486 M^{-1}), 223.0 nm (σ→π*, $\varepsilon_{223.0}$– 1438 M^{-1}), 269.5 nm (π→π*, $\varepsilon_{263.5}$ – 333 M^{-1}); IR (KBr, stretching frequency, cm^{-1}): 3360 (OH), 1261 (glycosidic aryl alkyl C-O-C asymmetrical), 1076 (glycosidic aryl alkyl C-O-C symmetrical), 1377 (C=C), 2937 (CH); MS (m/Z) 419$[M+1]^{+}$. 2D-HSQCT (DMSO-d_6) **C6-mannitol**: ^{1}H NMR δ_{ppm} (500.13) **Mannitol**: 3.61(H-6); **Bakuchiol**: 0.84 (H-17), 5.08 (H-18); ^{13}C NMR δ_{ppm} (125 MHz): **Mannitol**: 72.9 (C2), 70.4 (C3), 70.8 (C4), 72.9 (C5), 65.1 (C6); **Bakuchiol**: 38.2 (C10), 13.9 (C17).

4.3. Results and Discussion

Glycosylation of bakuchiol using conventional reflux method resulted in glycosides of D-glucose, D-ribose and D-arabinose only (Table 4.2) with yields in the range 9 - 51.4 %. Reactions carried out under supercritical CO_2 medium resulted in glycosides with aldohexoses – D-glucose, D-galactose and D-mannose; ketohexose – D-fructose; pentoses – D-ribose and D-arabinose; sugar alcohol – D-sorbitol and D-mannitol. The yields of the glycosides formed under $SCCO_2$ conditions were in the range 9 - 46.6% (Table 4.3).

Synthesis of bakuchiol glycosides with carbohydrate molecules showed that except for D-glucose, D-ribose and D-arabinose, the other carbohydrate molecules D-fructose, maltose, sucrose, lactose, D-sorbitol and D-mannitol did not undergo glycosylation under the conventional reflux conditions employed. This could be due to not-so-facile formation of the required oxocarbenium-ion intermediate [Moon and Tearo, 1998] with the other carbohydrate molecules at 68°C. Since the process conditions under SC-CO2 media are mild, they served as ideal conditions for the formation of glycosides with many carbohydrates. Glycosylation resulted in enhancement of water solubility of bakuchiol.

4.3.1. Spectral characterization

2DHSQCT spectrum of the standard bakuchiol is shown in Figure 4.3. Bakuchiol glycosides were characterized by UV, IR, Mass, Optical rotation and 2DHSQCT NMR (Fig. 4.4 to Fig 4.14). UV spectra of bakuchiol glycosides showed shifts in the $\sigma\rightarrow\sigma^*$band between 191.0nm to 191.5nm, $\sigma\rightarrow\pi^*$ band at 199.5nm to 231.5nm, $\pi\rightarrow\pi^*$ band at 259.5nm to 275nm and $n\rightarrow\pi^*$ band at 288.5nm. IR spectra showed shifts in the OH stretching frequency regain 2925 cm^{-1} - 3397 cm^{-1}, C=C at 1347 cm^{-1} – 1440 cm^{-1}, C-O-C asymmetrical at 1239 cm^{-1} – 1380 cm^{-1}, C-O-C symmetrical stretching at 1049 cm^{-1} – 1085 cm^{-1} and CH at 2923 cm^{-1} – 2937 cm^{-1}. 2DHSQCT NMR confirmed the formation of anomeric C1α and C1β products as well as C6 arylated products, especially C1 and C6 arylated products of D-sorbitol and D-mannitol.

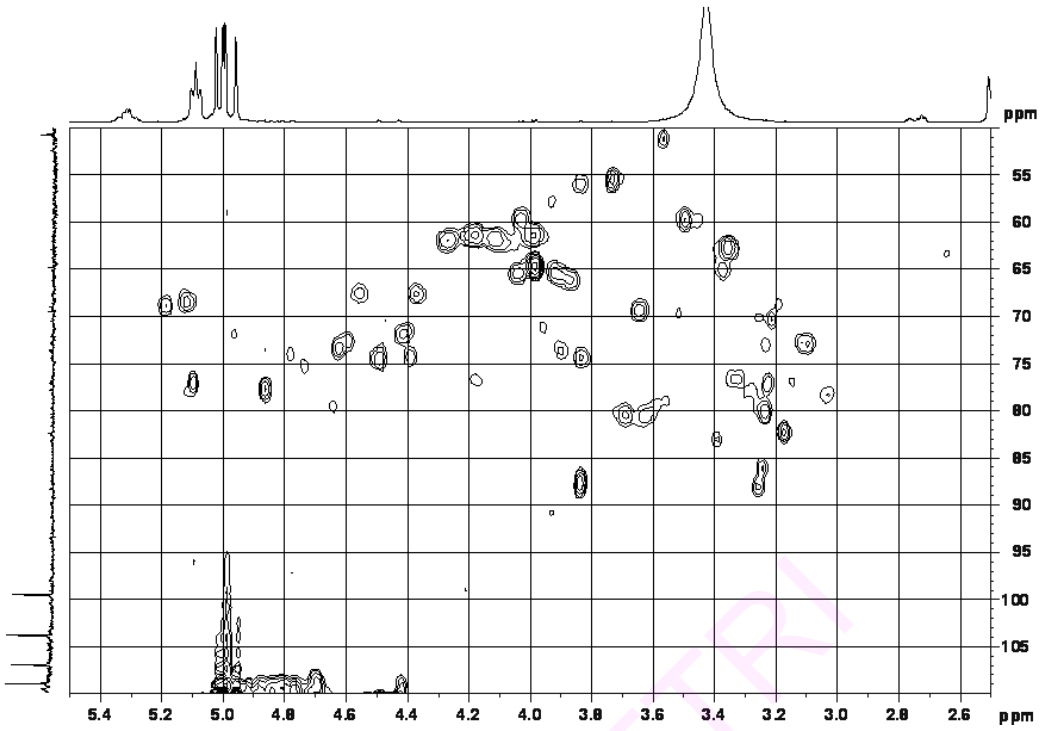


Fig. 4.3. Bakuchil standard. 2D-HSQCT spectrum showing the C1-C6 region. Some of the NMR assignments are interchangeable.

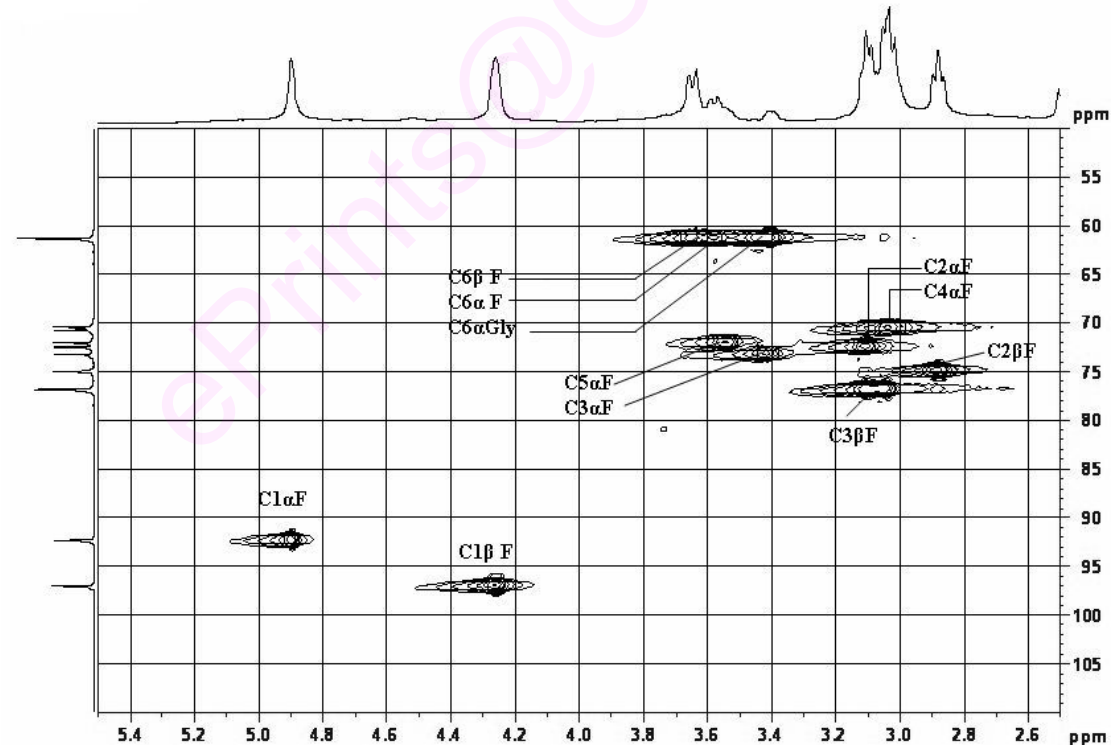


Fig. 4.4. 6-*O*-(D-Glucopyranosyl)bakuchiol by conventional method 2D-HSQCT spectrum showing the C1-C6 region. Some of the NMR assignments are interchangeable.

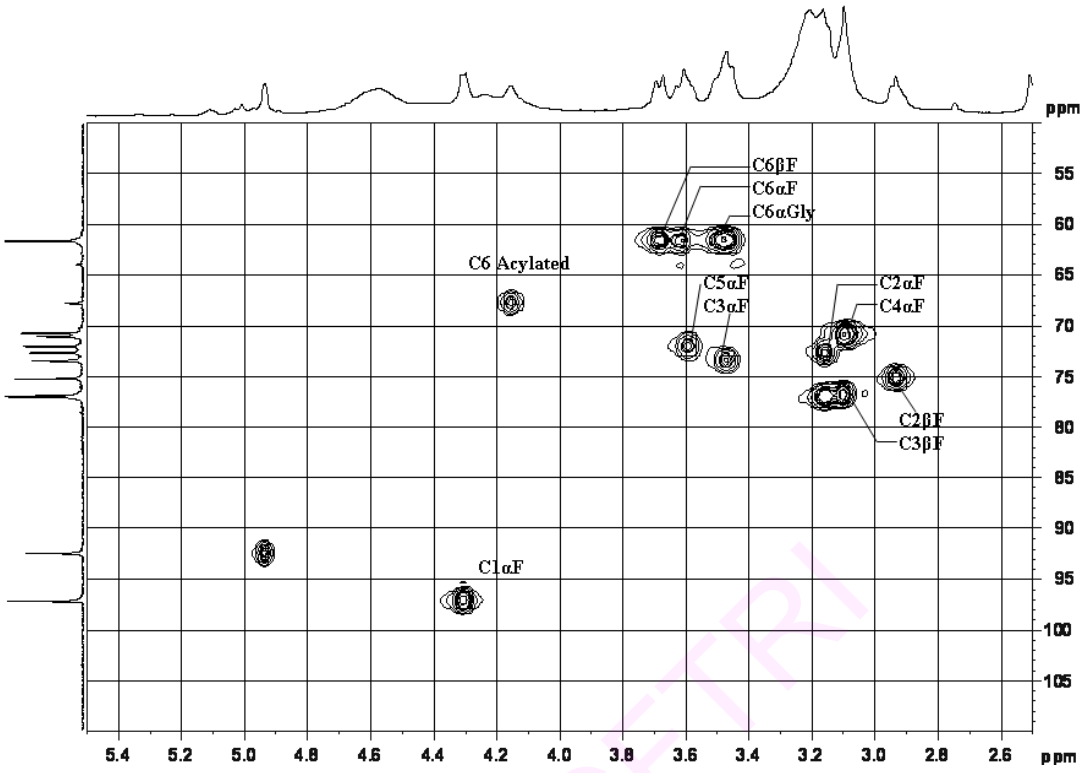


Fig. 4.5. 6-*O*-(D-Glucopyranosyl)bakuchiol in supercritical CO2 media 2D-HSQCT spectrum showing the C1-C6 region. Some of the NMR assignments are interchangeable.

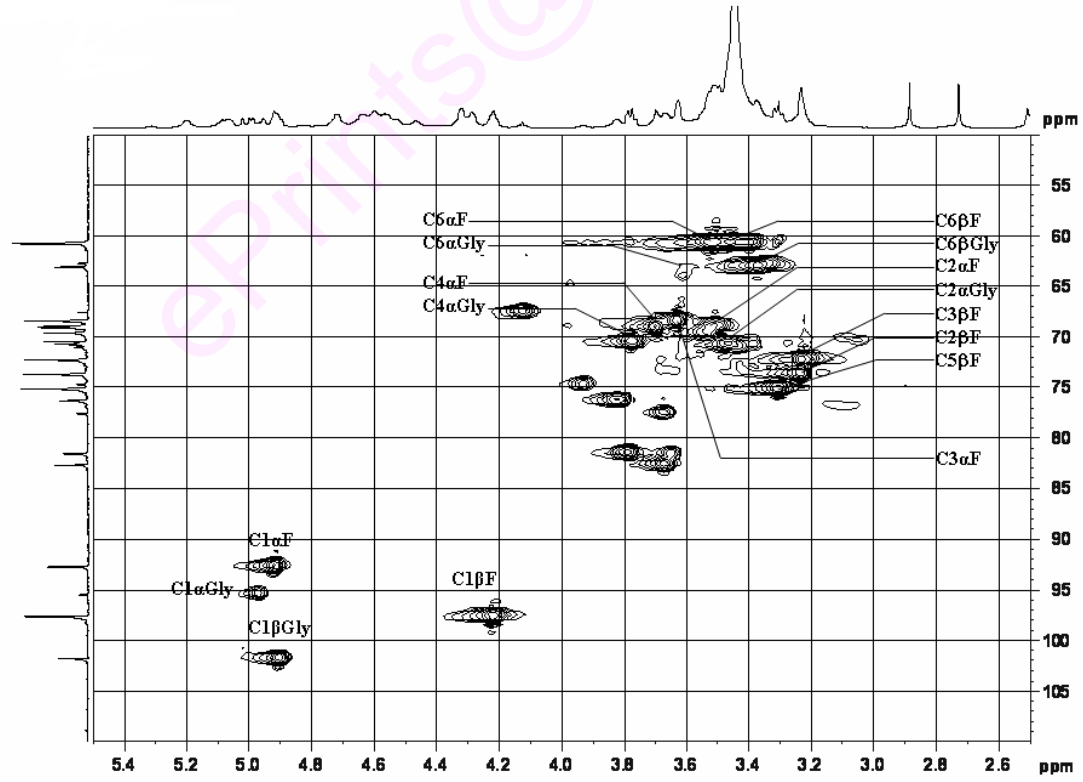


Fig. 4.6. 6-*O*-(D-Galactopyranosyl)bakuchiol in supercritical CO2 media 2D-HSQCT spectrum showing the C1-C6 region. Some of the NMR assignments are interchangeable.

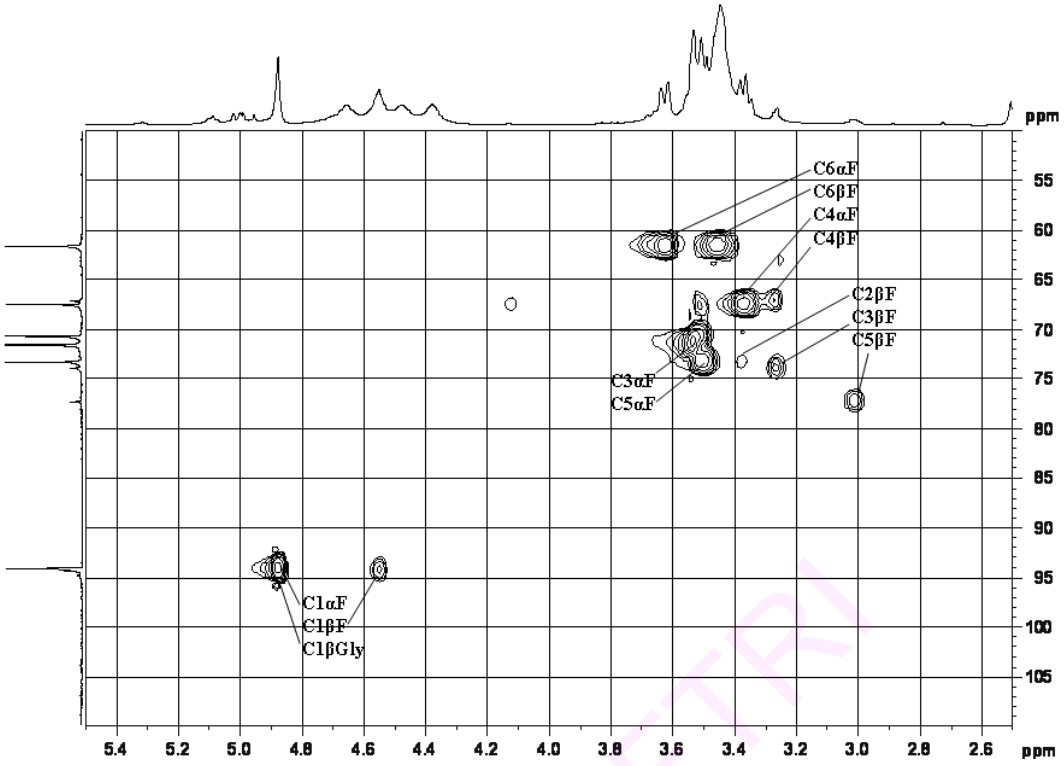


Fig. 4.7. 6-*O*-(D-Mannopyranosyl)bakuchiol in supercritical CO2 media 2D-HSQCT spectrum showing the C1-C6 region. Some of the NMR assignments are interchangeable.

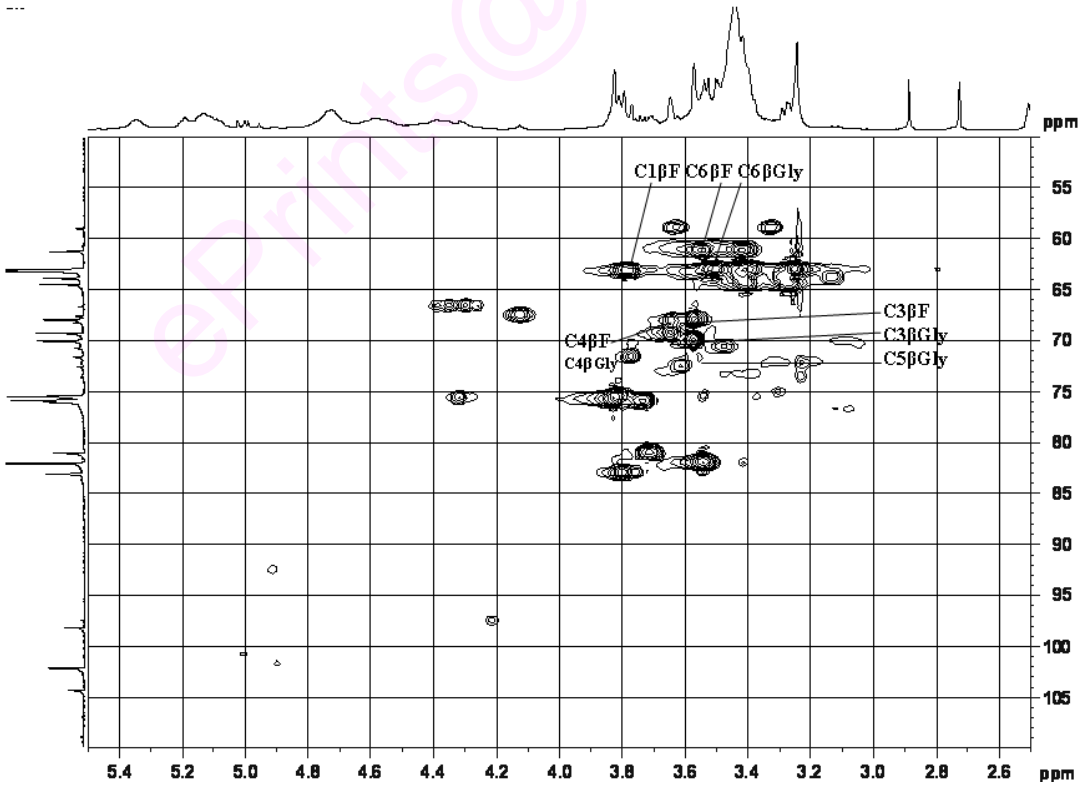


Fig.4.8. 6-*O*-(D-Fructofuranosyl)bakuchiol in supercritical CO2 media 2D-HSQCT spectrum showing the C1-C6 region. Some of the NMR assignments are interchangeable.

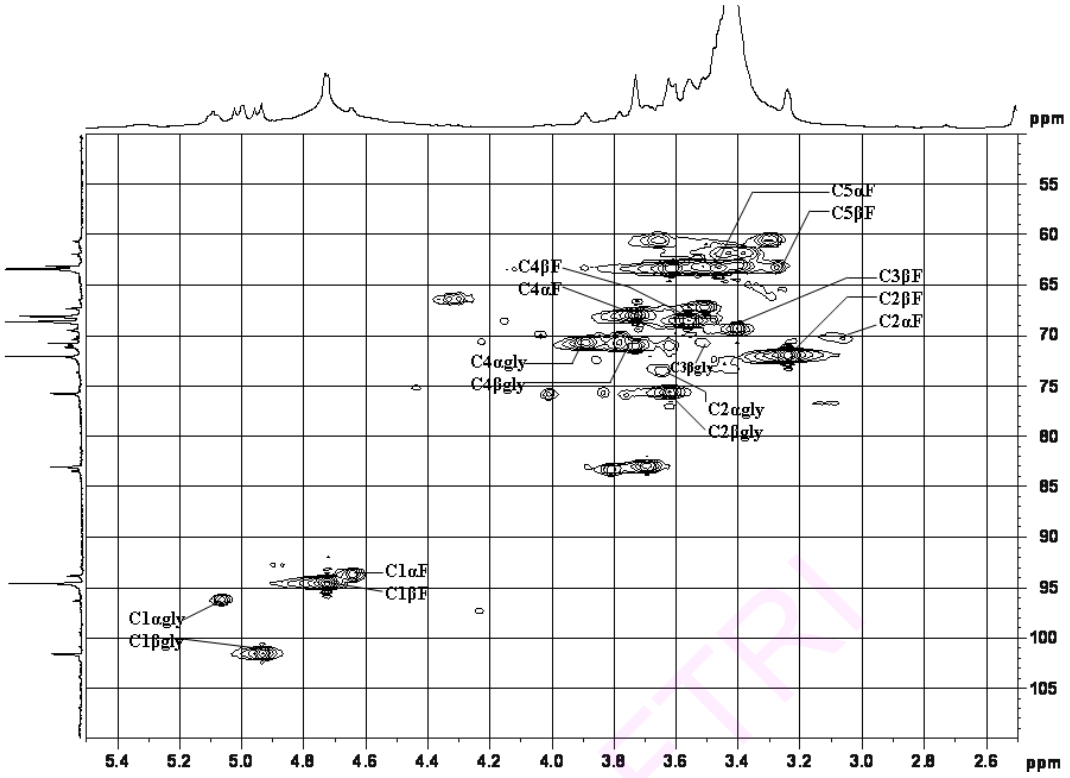


Fig. 4.9. 6-*O*-(D-Ribofuranosyl)bakuchiol by conventional method 2D-HSQCT spectrum showing the C1-C6 region. Some of the NMR assignments are interchangeable.

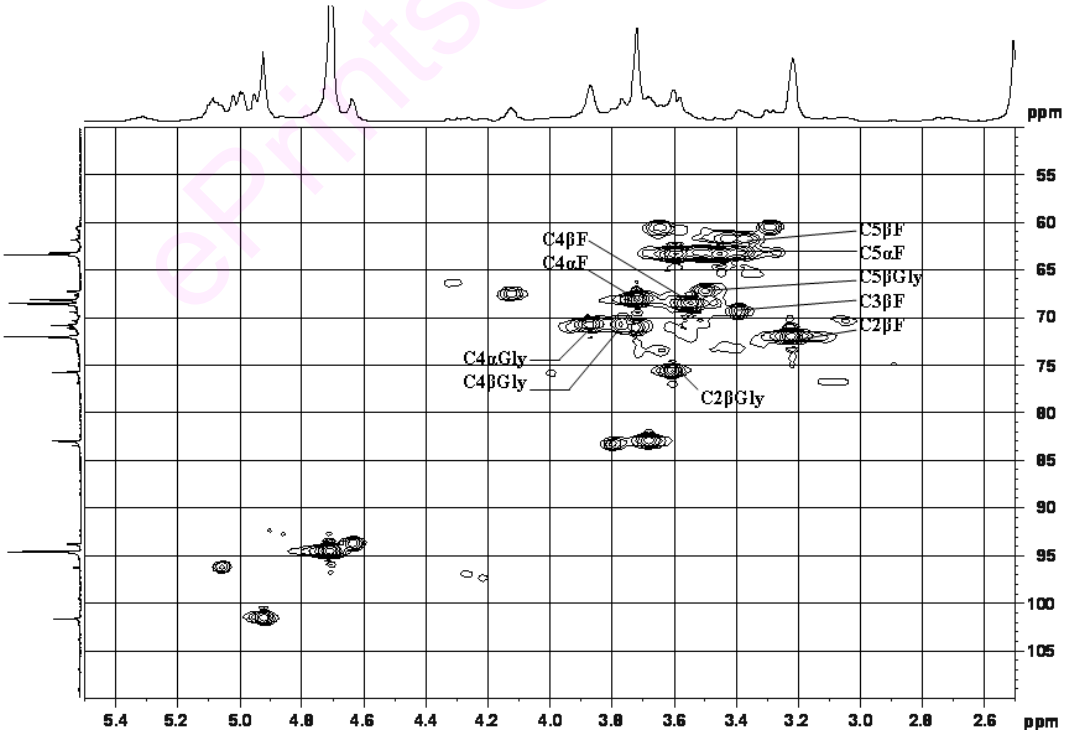


Fig. 4.10. 6-*O*-(D-Ribofuranosyl)bakuchiol in supercritical CO_2 media 2D-HSQCT spectrum showing the C1-C6 region. Some of the NMR assignments are interchangeable.

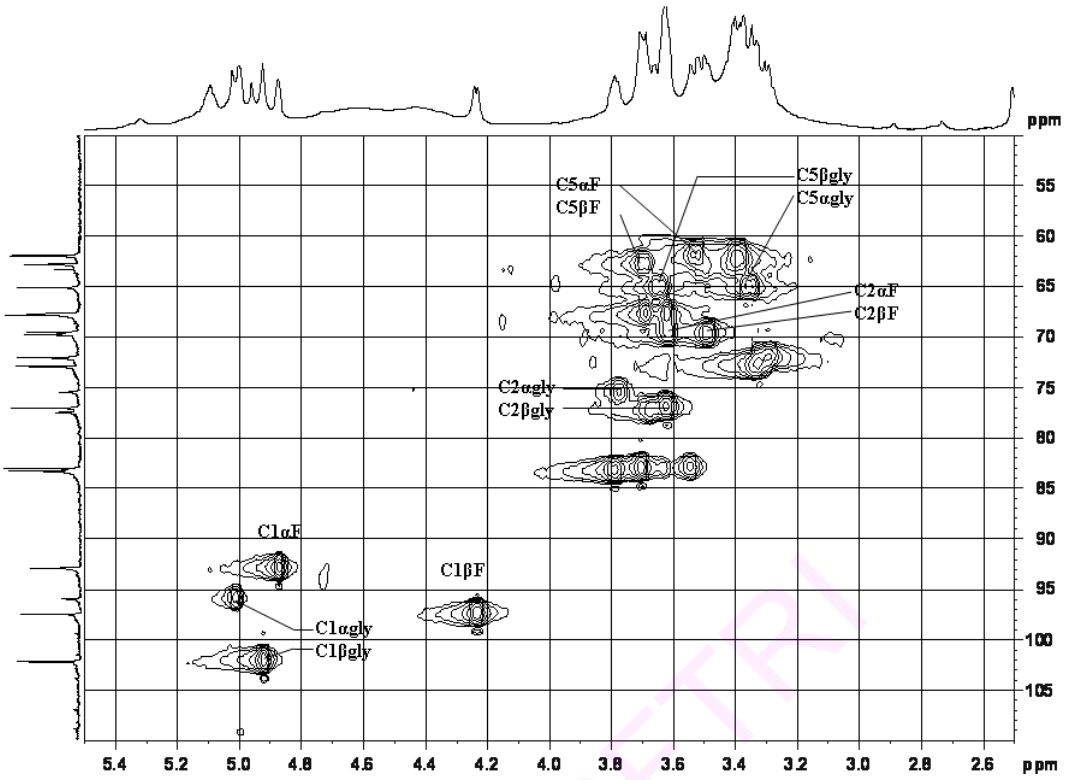


Fig.4.11. 6-*O*-(D-Arabinofuranosyl)bakuchiol by conventional method 2D-HSQCT spectrum showing the C1-C6 region. Some of the NMR assignments are interchangeable.

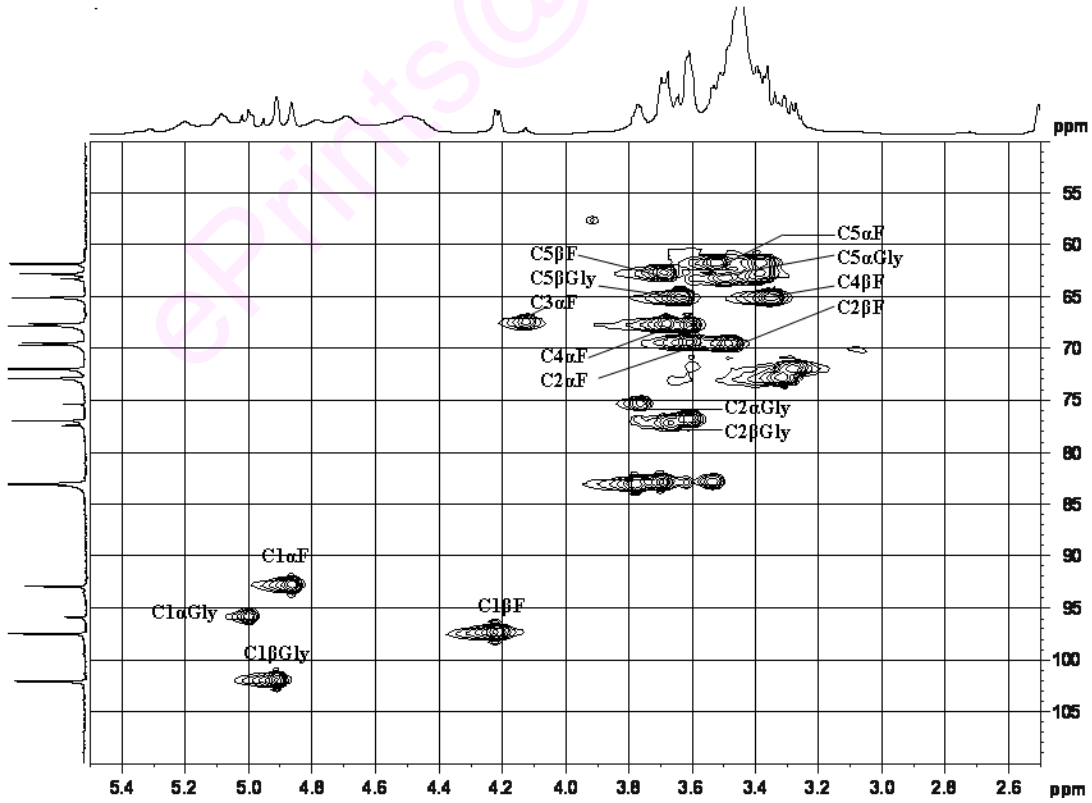


Fig. 4.12. 6-*O*-(D-Arabinofuranosyl)bakuchiol in supercritical CO2 media 2D-HSQCT spectrum showing the C1-C6 region. Some of the NMR assignments are interchangeable.

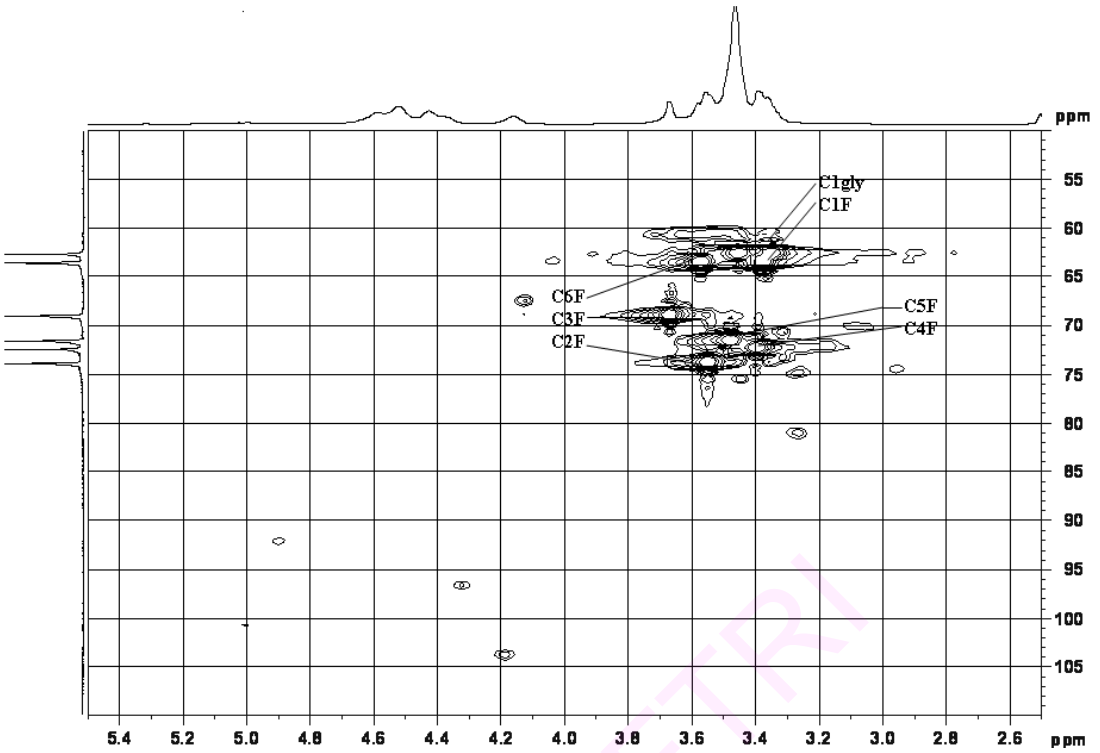


Fig. 4.13. 6-*O*-(1-D-Sorbitol)bakuchiol in supercritical CO2 media 2D-HSQCT spectrum showing the C1-C6 region. Some of the NMR assignments are interchangeable.

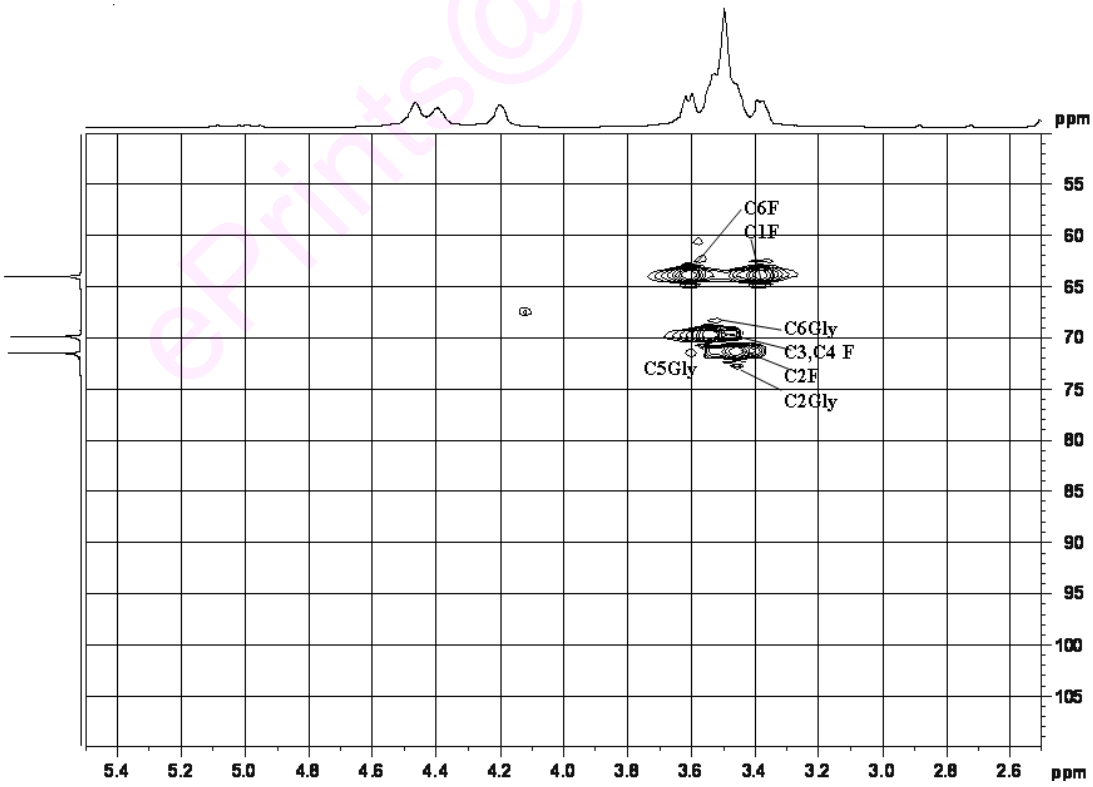


Fig. 4.14. 6-*O*-(6-D-Mannitol)bakuchiol in supercritical CO2 media 2D-HSQCT spectrum showing the C1-C6 region. Some of the NMR assignments are interchangeable.

Wherever glycosides formed with bakuchiol, HPLC chromatograms of the bakuchiol glycosides prepared by conventional reflux method and supercritical CO_2 glycosylation method are shown (Figures 4.15 to 4.25). Table 4.4 gives relative retention time of bakuchiol glycosides (prepared at $SCCO_2$ conditions) with respect to retention time of standard glucose.

4.3.2. Antioxidant activity

Antioxidant activities of glycosides of bakuchiol are presented in Table 4.4. Pure bakuchiol showed an antioxidant activity of 1.24 mM (IC_{50} value) as against 0.029 mM for synthetic antioxidant BHA. Various glycosides of bakuchiol showed antioxidant activities ranging from 1.02 to 2.28 mM. Among the 8 glycosides prepared 6-*O*-(D-ribofuranosyl)bakuchiol and 6-*O*-(D-arabinofuranosyl)bakuchiol showed very low IC_{50} values of 1.02 ± 0.102mM and 1.2±0.12mM, while 6-*O*-(D-galactopyranosyl) bakuchiol (1.28 ± 0.128mM) and 6-*O*-(D-glucopyranosyl) bakuchiol (1.34 ± 0.134mM) showed significant IC_{50} values for antioxidant activity. Carbohydrate molecules themselves did not show antioxidant activities. Although phenolic OH group of bakuchiol is modified, it still showed marginally better antioxidant activity better than bakuchiol itself.

4.3.3. ACE inhibition

Bakuchiol glycosides were also tested for ACE inhibition. ACE inhibitory activities of bakuchiol glycosides are presented in Table 4.5. Bakuchiol glycosides exhibited almost lesser IC_{50} values for ACE inhibition than bakuchiol itself. Among the different glycosides prepared, 6-*O*-(6-D-fructofuranosyl)bakuchiol, 0.64 ± 0.06mM6-*O*-(D-ribofuranosyl)bakuchiol, 0.85±0.09mM,6-*O*-(D-mannopyranosyl) bakuchiol, 0.85 ± 0.09mM and 6-*O*-(6-D-mannitol)bakuchiol, 0.89 ± 0.09 mM exhibited better IC_{50} values than the other glycosides. 6-*O*-(D-Arabinofuranosyl) bakuchiol, 1.03 ± 0.10mM, 6-*O*-(1-D-sorbitol)bakuchiol, 1.20 ± 0.12mM, 6-*O*-(D-galactopyranosyl)bakuchiol, 1.22 ± 0.12mM and 6-*O*-(D-glucopyranosyl)bakuchiol, 1.33 ± 0.13mM showed high IC_{50} values for ACE inhibition. Bakuchiol and enalapril showed IC_{50} values of 0.74 ± 0.07mM and 0.071 ± 0.007mM for ACE inhibition respectively. 6-*O*-(6-D-Fructofuranosyl) bakuchiol with IC_{50} value of 0.64± 0.06mM

has shown the best ACE inhibition than bakuchiol itself. Modification of the phenolic OH group by the carbohydrate molecule did not affect the ACE inhibition activity.

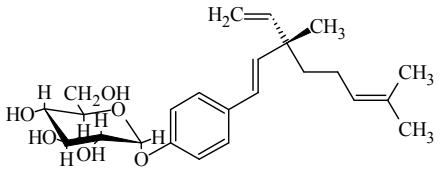
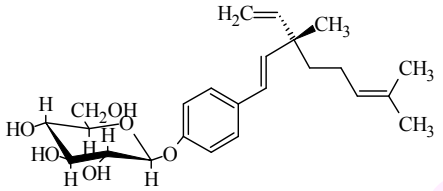
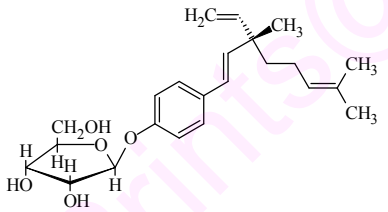
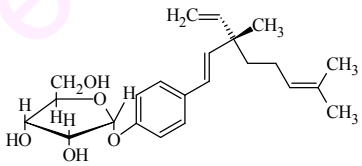
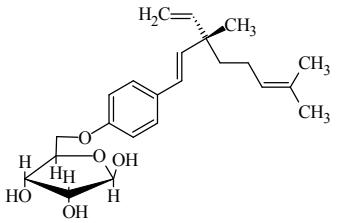
In contrast, under the supercritical CO_2 atmosphere, glycosides with carbohydrate molecules of carbohydrates D-glucose, D-fructose, D-ribose, D-sorbitol, D-arabinose, D-mannose, and D-mannitol were formed and three disaccharides of maltose, sucrose and lactose were not detected. This could be due to the usefulness of the reaction medium which provided an ideal dielectric medium for the enzymatic reaction to occur with wide variety of carbohydrates. The yield of glycosides were in the range of 9- 46.6%.

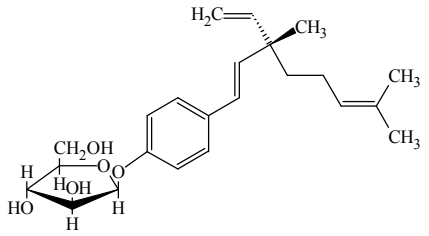
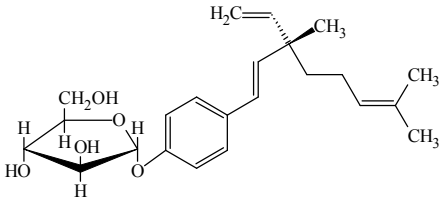
Among the various carbohydrate molecules employed, particularly the glycosylation of aldo-hexoses like D-glucose, D-galactose and ketohexose D-fructose, aldo-pentoses like D-ribose, D-arabinose and sugar alcohol D-mannitol with phenolic OH group of bakuchiol converted bakuchiol into a freely water soluble compounds as well as enhance its biological activities also.

4.4. Conclusions

Enzymatic syntheses of water soluble bakuchiol glycosides were reported first time. The reactions were carried in two different methods: one by conventional reflux method and the other under supercritical CO_2 atmospheric conditions. Out of eleven carbohydrates selected for the reaction, D-glucose, D-ribose and D-arabinose gave glycosides in yields of 9.0% to 51.4% under conventional reflux method. Under $SCCO_2$ atmosphere, bakuchiol formed glycosides with D-glucose, D-galactose, D-mannose, D-fructose, D-ribose, D-arabinose, D-sorbitol and D-mannitol in yield range 9% to 46.6%. Antioxidant and ACE inhibitory activity of bakuchiol glucosides showed that 6-O-(6-D-Fructofruranosyl) bakuchiol showed the best antioxidant (1.4mM) and ACE inhibitory activities (0.64 mM).

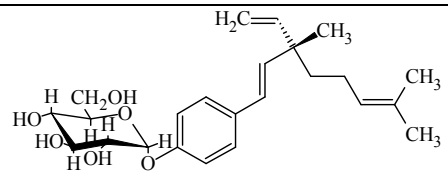
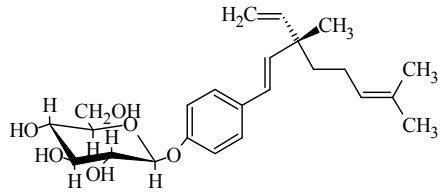
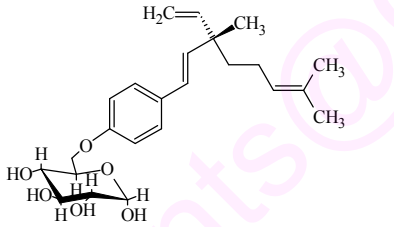
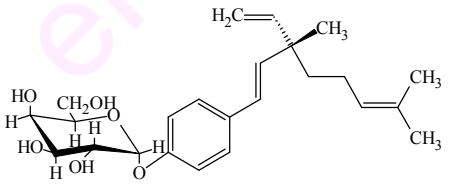
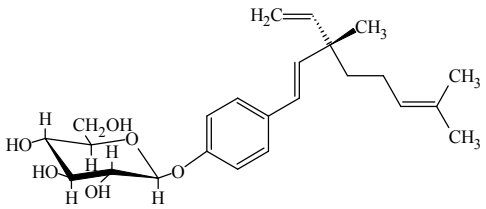
Table 4.2. Conversion yields and proportions of bakuchiol glycosides prepared by the conventional reflux method[a]

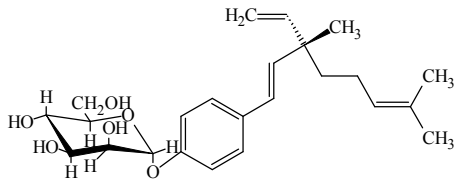
No.	Glycosides	Amyloglucosidase catalysis[b]	
		Product (% proportions)[c]	Yield (%)[d]
1.		6-*O*-α (45)	
	6-*O*-(α-D-Glucopyranosyl)bakuchiol		9.0
		6-*O*-β (55)	
	6-*O*-(β-α-D-Glucopyranosyl)bakuchiol		
2			
	6-*O*-(α-D-Ribofuranosyl)bakuchiol	6-*O*-α (23)	
			51.4
	6-*O*-(β-D-Ribofuranosyl)bakuchiol	6-*O*-β (53)	
			
	6-*O*-(5-D-Ribofuranosyl)bakuchiol	6-*O*-5 (24)	

3	 **6-*O*-(α-D-Arabinofuranosyl)bakuchiol**	6-*O*-α (27)	42.0
	 **6-*O*-(β-D-Arabinofuranosyl)bakuchiol**	6-*O*-β (73)	

[a]Reaction refluxed at 68°C in di-isopropyl ether solvent at atmospheric pressure; [b]Bakuchiol – 0.5 m.mol and carbohydrate 1.0m.mol; enzyme concentration 40% w/w carbohydrates; solvent – di-isopropyl ether, DMF – 5.0 ml; 0.1 mM (1.0ml) pH 6.0 phosphate buffer; incubation period – 72; [c]The product proportions were calculated from the area of respective carbon signals. [d]Conversion yields were from HPLC with respect to the carbohydrate. Error in yield measurements is ± 10% .

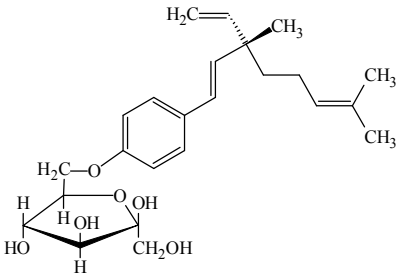
Table 4.3. Conversion yields and proportions of bakuchiol glycosides prepared under $SCCO_2$ condition.[a]

No.	Glycosides	Amyloglucosidase catalysis[b]	
		Product (% proportions)[c]	Yield (%)[d]
1		6-*O*-α (17)	
	6-*O*-(α-D-Glucopyranosyl)bakuchiol		
		6-*O*-β (22)	9
	6-*O*-(β-D-Glucopyranosyl)bakuchiol		
		6-0-6 arylated (61)	
	6-*O*-(5-D-Glucopyranosyl)bakuchiol		
2.		6-*O*-α (29)	
	6-*O*-(α-D-Galactopyranosyl)bakuchiol		
		6-*O*-β (71)	37.2
	6-*O*-(β-D- Galactopyranosyl)bakuchiol		

3. 

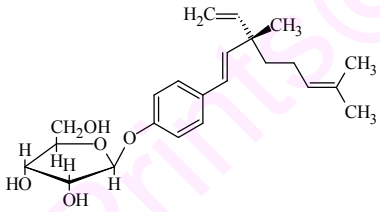
6-*O*-α 32.7

6-*O*-(α-D-Mannoopyranosyl)bakuchiol

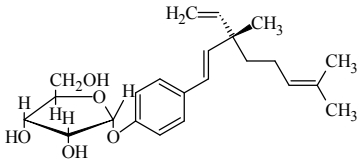
4. 

6-*O*-6 31.0

6-*O*-(6-D-Fructofuranosyl)bakuchiol

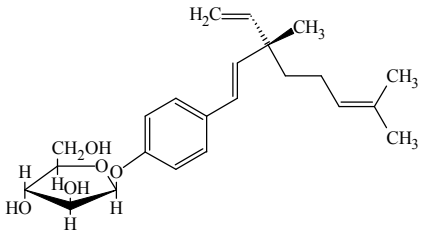
5. 

6-*O*-(α-D-Ribofuranosyl)bakuchiol 6-*O*-α (31) 33.3



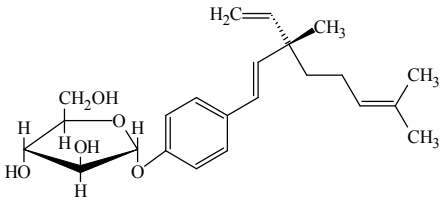
6-*O*-β (69)

6-*O*-(β-D-Ribofuranosyl)bakuchiol

6. 

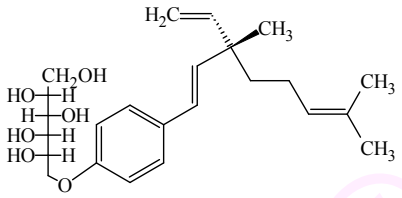
6-*O*-(38) 38

6-*O*-(α-D-Arabinofuranosyl)bakuchiol



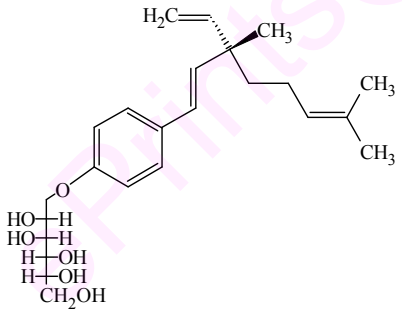
6-*O*-β (62)

6-*O*-(β-D-Arabinofuranosyl)bakuchiol

7. 

6-*O*-1 46.6

6-*O*-(1-D-Sorbitol)bakuchiol

8. 

6-*O*-6 29.15

6-*O*-(6-D-Mannitol)bakuchiol

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[a]Reaction conducted under 100 bar CO_2 at 50°C. [b]Bakuchiol – 0.5 m.mol and carbohydrate 1.0m.mol; enzyme concentration 40% w/w carbohydrates; solvent – DMF – 15.0 ml; 0.1 mM (1.0ml) pH 6.0 phosphate buffer; incubation period – 24. [c]The product proportions were calculated from the area of respective carbon signals. [d]Conversion yields were from HPLC with respect to the carbohydrate. Error in yield measurements is ± 10% .

Table 4.4. IC_{50} values for Antioxidant activities of bakuchiol glycosides

Compound	IC_{50} value (mM)
Butylated Hydroxy Anisole	0.029 ± 0.003
Bakuchiol	1.24 ± 0.12
6-*O*-(D-Glucopyranosyl)bakuchiol	1.34 ± 0.13
6-*O*-(D-Galactopyranosyl)bakuchiol	1.28 ± 0.13
6-*O*-(D-Mannoopyranosyl)bakuchiol	2.13 ± 0.21
6-*O*-(6-D-Fructofuranosyl)bakuchiol	1.40 ± 0.14
6-*O*-(D-Ribofuranosyl)bakuchiol	1.02 ± 0.10
6-*O*-(D-Arabinofuranosyl)bakuchiol	1.20 ± 0.12
6-*O*-(1-D-Sorbitol)bakuchiol	2.28 ± 0.23
6-*O*-(6-D-Mannitol)bakuchiol	1.80 ± 0.18

Table 4.5. IC_{50} values for Angiotensin converting enzyme inhibitory activities of bakuchiol glycosides

Compound	IC_{50} value (mM)
Enalapril	0.071± 0.007
Bakuchiol	0.74 ± 0.07
6-*O*-(D-Glucopyranosyl)bakuchiol	1.33 ± 0.13
6-*O*-(D-Galactopyranosyl)bakuchiol	1.22 ± 0.12
6-*O*-(D-Mannoopyranosyl)bakuchiol	0.85 ± 0.09
6-*O*-(6-D-Fructofuranosyl)bakuchiol	0.64 ± 0.06
6-*O*-(D-Ribofuranosyl)bakuchiol	0.85 ± 0.09
6-*O*-(D-Arabinofuranosyl)bakuchiol	1.03 ± 0.10
6-*O*-(1-D-Sorbitol)bakuchiol	1.20 ± 0.12
6-*O*-(6-D-Mannitol)bakuchiol	0.89 ± 0.09

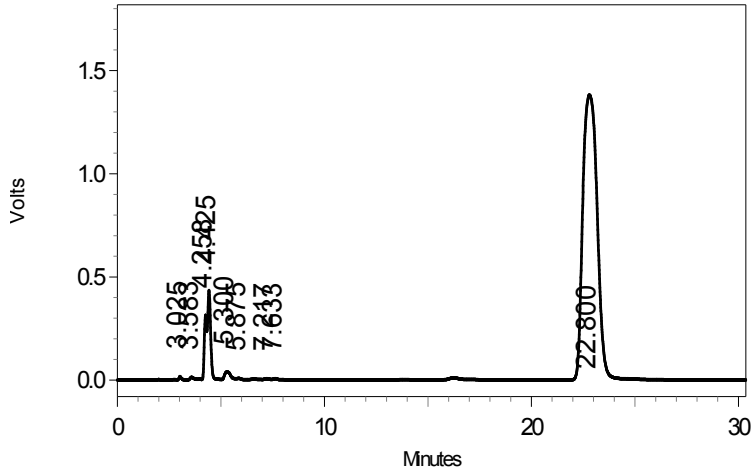

Fig. 4.15. HPLC chromatogram of 6-*O*-(D-Glucopyranosyl)bakuchiol prepared by conventional reflux method.

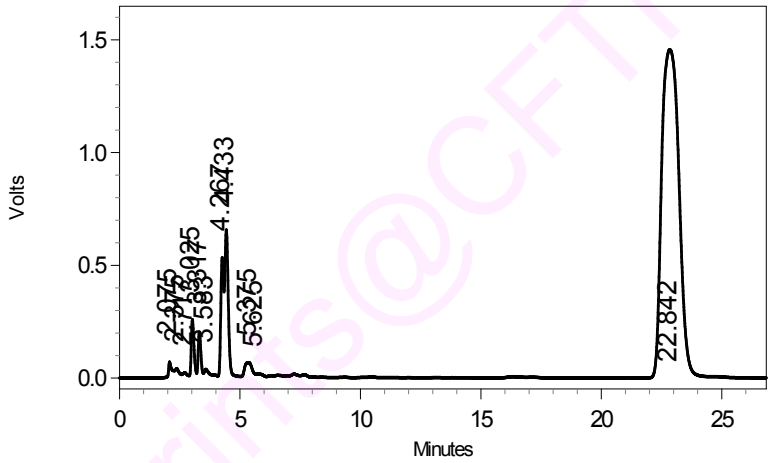

Fig. 4.16. HPLC chromatogram of 6-*O*-(D-Ribofuranosyl)bakuchiol prepared by conventional reflux method.

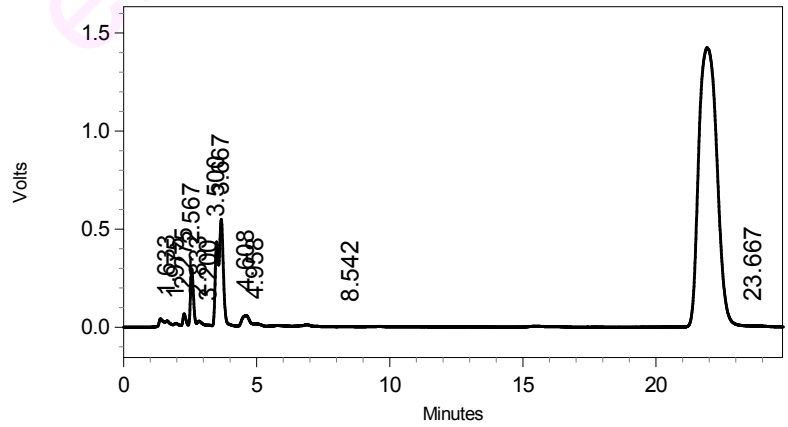

Fig. 4.17. HPLC chromatogram of 6-*O*-(α-D-Arabinofuranosyl)bakuchiol prepared by conventional reflux method.

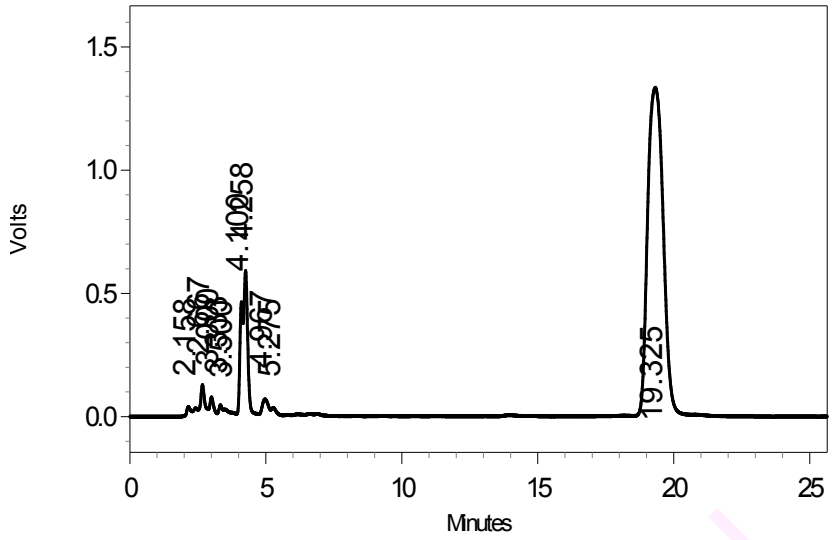

Fig. 4.18. HPLC chromatogram of 6-*O*-(D-Glucopyranosyl)bakuchiol prepared at supercritical CO_2 conditions.

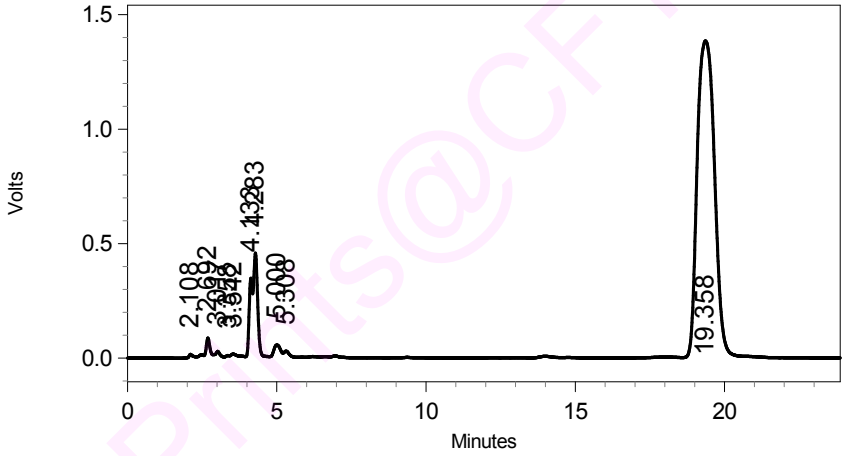

Fig. 4.19. HPLC chromatogram of 6-*O*-(D-Galactopyranosyl)bakuchiol prepared at supercritical CO_2 conditions.

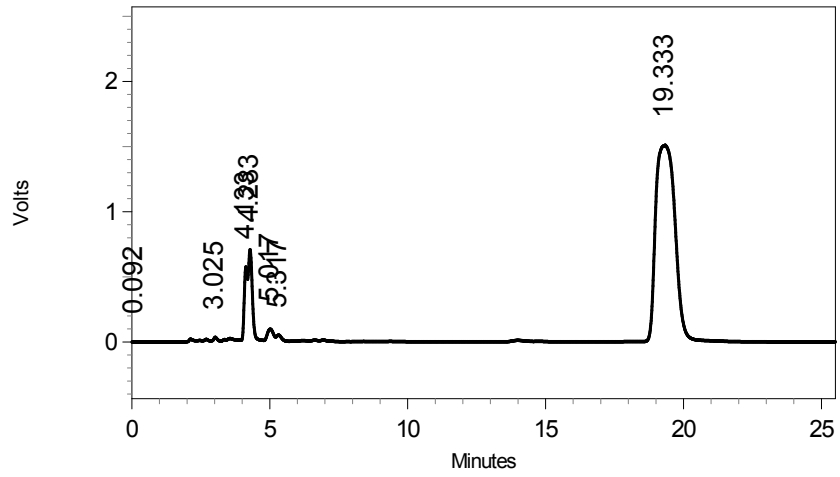

Fig. 4.20. HPLC chromatogram of 6-*O*-(D-Mannopyranosyl)bakuchiol prepared at supercritical CO_2 conditions.

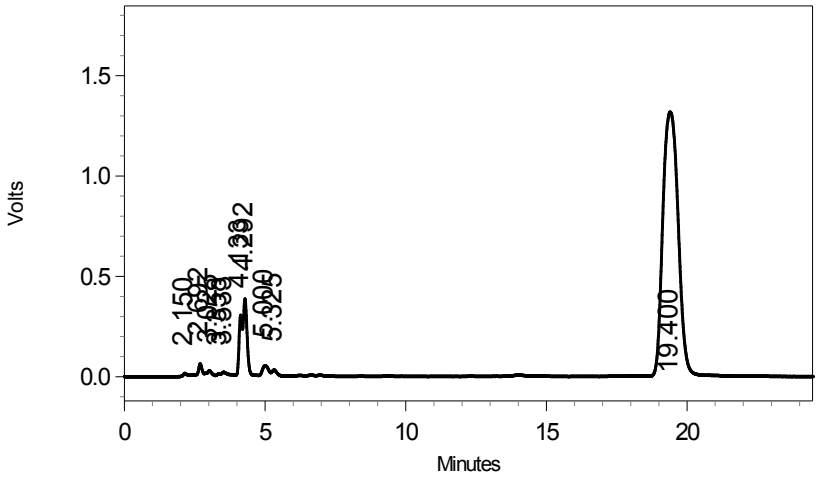

Fig. 4.21. HPLC chromatogram of 6-*O*-(D-Fructofuranosyl)bakuchiol prepared at supercritical CO_2 conditions.

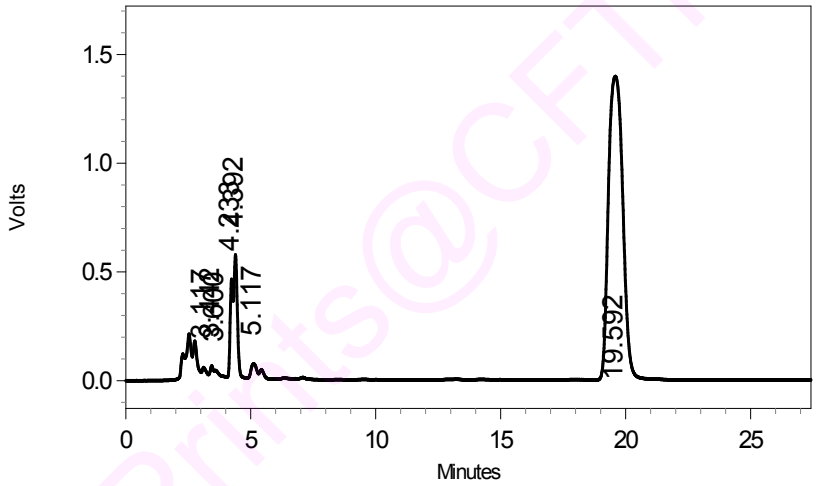

Fig. 4.22. HPLC chromatogram of 6-*O*-(D-Ribofuranosyl)bakuchiol prepared at supercritical CO_2 conditions.

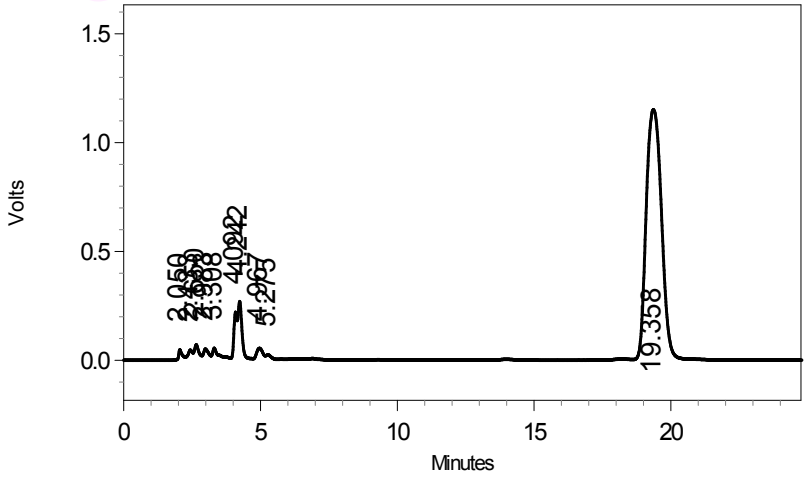

Fig. 4.23. HPLC chromatogram of 6-*O*-(D-Arabinofuranosyl)bakuchiol prepared at supercritical CO_2 conditions.

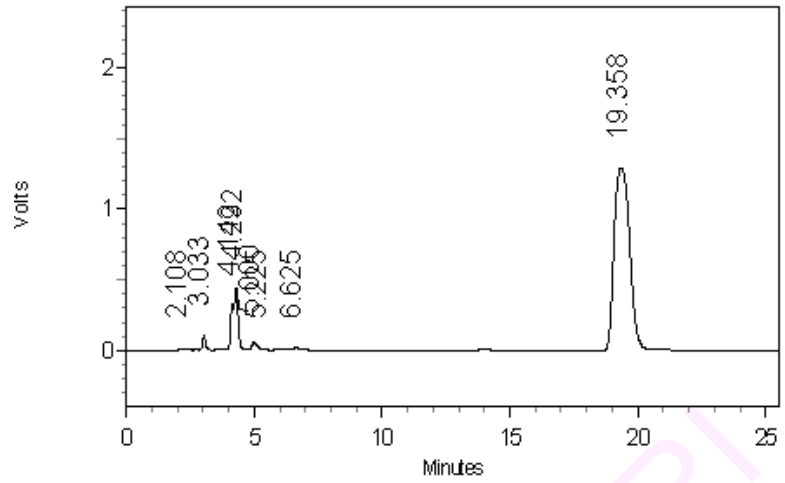

Fig. 4.24. HPLC chromatogram of 6-*O*-(1-D-Sorbitol)bakuchiol prepared at supercritical CO_2 conditions.

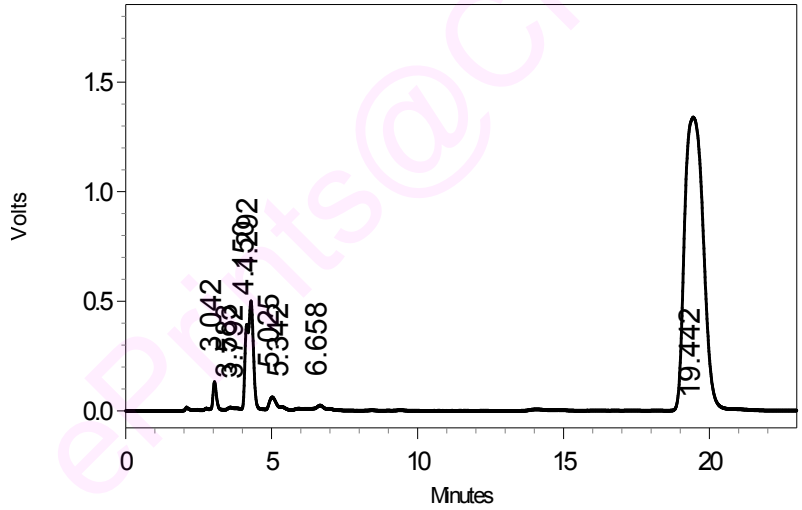

Fig. 4.25. HPLC chromatogram of 6-*O*-(6-D-Mannitol)bakuchiol prepared at supercritical CO_2 conditions.

SUMMARY

The rapid emergence of nutraceuticals and functional foods has created a new trend in food processing industries. The nutraceutical and functional foods are more often prepared from the phytochemicals / plant extracts. They are gaining popularity because of several advantages such as fewer adverse health side effects, better patient tolerance, relatively low price and acceptance due to a long history of use. The more important cause is that natural products provide rational means for the treatment of many diseases that are obstinate and incurable.

Among the various plant materials available for extraction of bioactives, the present thesis focuses on *Psoralea corylifolia* L, known commonly as chiba or bakuchi. The main objectives of the thesis are: a) Preparation of plant extract that contains important bioactives like bakuchiol and psoralen using supercritical carbon dioxide ($SCCO_2$) extraction technology. b) Characterization of the extract in terms of important bioactives. c) Enrichment of the extract with higher concentration of bioactives by adopting high vacuum molecular distillation technology d) Enzymatic synthesis of water soluble bakuchiol glycosides and study of their anti-oxidant and Angiotensin Converting Enzyme (ACE) inhibitory activities. The thesis is divided in to four chapters listed below for easy reading and chapter-wise briefing is presented here.

Chapter 1. Introduction
Chapter 2. Supercritical fluid extraction and characterization of the extract
Chapter 3. Enrichment of bakuchiol using molecular distillation technique
Chapter 4. Enzymatic synthesis of bakuchiol glycosides

Chapter 1 presents information on importance of nutraceuticals, bioactive components of the *Psoralea corylifolia* L. and their biological activities supported by literature.

Chapter 2 deals with supercritical fluid extraction of bioactives from *Psoralea corylifolia* seeds. A brief review of literature on the supercritical fluid extraction is presented. The thesis reports, for the first time, the extraction of bioactives from chiba seeds using $SCCO_2$.

Extraction studies and modeling: The extraction of bioactives from *Psoralea corylifolia* seeds was carried out using the high pressure $SCCO_2$ system at pressures

220, 260 and 300 bar and at temperature of 40°C. Even though yield of extraction by $SCCO_2$ extraction process was slightly less than the yield by hexane extraction method, bakuchiol concentration in the extract was much higher than the hexane extraction. The mass transfer of the extraction was modeled based on Diffusion model, Exponential model, Langmuir model and Logistic model and comparative evaluation of the models was done. LC-MS chromatogram of the extract identified presence of nine compounds. The results indicated that the extract having bakuchiol concentration of 51% was possible with $SCCO_2$ extraction.

Determination of critical properties: Critical properties like critical temperature, critical pressure, critical volume and normal boiling point which are important for solubility determination, process design and development were predicted for key components, bakuchiol and psoralen, based on four different group contribution methods such as Ambrose, Joback, Klincewicz-Reid and Lydersen methods. The predictions showed that there is substantial difference between the vapor pressure of bakuchiol and psoralen.

Prediction of solubility: The solubility of bakuchiol and psoralen at 220, 260 and 300 bar was predicted based on equation of state models Peng-Robinson (PR) and Soave-Redlich-Kwong (SRK). The solubilties (in mole fraction) of bakuchiol and psoralen were found to be in the range 10^{-6} to 10^{-4}. The models indicated high enhancement factors for both the components implying high non-ideal behavior. The predicted solubility agreed reasonably well with experimental values of solubilities for bakuchiol and psoralen.

Chapter 3 is a study on the molecular distillation technique to increase the bakuchiol content in the extract obtained by $SCCO_2$. The effect of process variables such as vacuum (400-2000 μm), temperature 140–200 °C) and wiper speed (120–200 RPM) on the main bioactive component bakuchiol was studied based on Response Surface Methodology (RSM) following 15-point Box-Behnken design of experiment. A predicted maximum of 72% bakuchiol was possible under the optimized process conditions and the same was confirmed by the experiment. The study established that the separation and concentration of bakuchiol from the extract was possible using molecular distillation technique without degradation / cyclisation of bakuchiol.

Chapter 4 deals with importance of glycosides, enzymatic synthesis of bakuchiol glycosides with various carbohydrates and their anti-oxidant and Angiotensin Converting Enzyme (ACE) inhibition properties. Bakuchiol exhibits poor water solubility, stability and absorbability. Glycosylation improves the pharmacological property by increasing the water solubility of bakuchiol. Enzymatic synthesis of water soluble bakuchiol glycosides is reported for the first time in this study using amyloglucosidase from *Rhizopus* mold carried out in two different media: one by conventional reflux method and the other under supercritical CO_2 atmospheric conditions. Out of eleven carbohydrate molecules selected for the reaction, D-glucose, D-ribose and D-arabinose gave glycosides in yields of 9.0% to 51.4% under conventional reflux method. Under supercritical CO_2 atmosphere (100 bar pressure at 50°C), bakuchiol formed glycosides with D-glucose, D-galactose, D-mannose, D-fructose, D-ribose, D-arabinose, D-sorbitol and D-mannitol in yield range 9% to 46.6%. Antioxidant and ACE inhibitory activity of bakuchiol as well as bakuchiol glycosides were determined.

Thus the investigations presented clearly brought out the extractability of major bioactives from *P. corylifolia* using $SCCO_2$, enrichment of the extract using molecular distillation and preparation of water soluble glycosides to enhance the bioavailability and bioactivity of the bioactive components of *P. corylifolia*.

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Research papers published based on the thesis

Published in international peer reviewed journals:

1. **B. Manohar** and K. Udaya Sankar (2009). Enrichment of Bakuchiol in Supercritical Carbon Dioxide Extracts of Chiba Seed (*Psoralea corylifolia* L.) Using Molecular Distillation – Response Surface Methodology. **Biotechnology and Bioprocess Engineering**, 14: 112-117.

2. **Balaraman Manohar,** Soundar Divakar and Kadimi Udaya Sankar. (2009). Amyloglucosidase catalyzed syntheses of Bakuchiol glycosides in supercritical carbon dioxide. **Bull. Korean Chem. Soc**., 30 (8): 1760-1766.

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