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Chemical and Microbiological Investigations of Cassia fistula Linn

Ali, Md. Abbas

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Chemical and Microbiological Investigations of *Cassia fistula* Linn.

Thesis Submitted to the Department of Applied Chemistry and Chemical Technology, University of Rajshahi, Bangladesh, in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy

By

Md. Abbas Ali

B. Sc. (Hons), M.Sc.

Protein & Enzyme Research Laboratory
Department of Biochemistry and Molecular Biology
University of Rajshahi, Bangladesh

Organic Research Laboratory
Department of Applied Chemistry and Chemical Technology, University of Rajshahi, Bangladesh

November, 2002

Dedicated to
My Beloved Daughter

DECLARATION

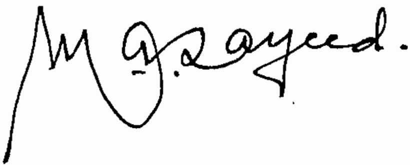
I do hereby declare that the materials embodied in this thesis entitled "Chemical and Microbiological Investigations of *Cassia fistula* Linn." prepared for submission to the University of Rajshahi, Bangladesh, for the Degree of Doctor of Philosophy in Applied Chemistry and Chemical Technology, are the original research works of mine and have not been concurrently submitted for any other degree or diploma.

(Md. Abbas Ali)
Signature of Candidate

CERTIFICATE

This is to certify that the thesis entitled "Chemical and Microbiological Investigations of *Cassia fistula* Linn." is an original research work done by Md. Abbas Ali for the Degree of Doctor of Philosophy under our supervision. It contains no material previously published or submitted for any other degree or diploma.

Supervisors:



1. **(Dr. Md. Abu Sayeed)**
Professor
Department of Applied Chemistry and Chemical Technology, University of Rajshahi, Bangladesh

2. **(Dr. Nurul Absar)**
Professor
Department of Biochemistry and Molecular Biology
University of Rajshahi, Bangladesh

Contents

	Page No.
Acknowledgement	I
Abstract	II
List of Table	V
List of Figure	X
List of Abbreviation	XIII
Chapter-1: General Introduction and Literature Review.	1
Chapter-2: Purification and Characterization of Proteins from *Cassia fistula* Seed.	15
Introduction	
Materials and Methods	
Results and Discussion	
Chapter-3:Effect of Physico-chemical Agents on the Hemagglutinating Activities of *Cassia fistula* Seed Lectins.	43
Introduction	
Materials and Methods	
Results and Discussion	
Chapter-4: Physico-chemical Investigations of *Cassia fistula* Seed oil	53
Introduction	
Materials and Methods	
Results and Discussion	
Chapter-5: Isolation, Purification and Characterization of Compounds from *Cassia fistula* Stem Bark.	70
Introduction	
Materials and Methods	
Results and Discussion	

Chapter-6: Biological Investigations on *Cassia fistula*.

Introduction 97

Materials and Methods

Results and Discussion

Chapter-7: Biochemical Analysis of *Cassia fistula* Seeds and Leaves. 126

Introduction

Materials and Methods

Results and Discussion

Chapter-8: Conclusion 136

References 140

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The Author

Abstract

Three lectins were extracted and purified from *Cassia fistula* seeds in biologically active form by gel filtration of 100 % ammonium sulfate saturated crude protein extract on Sephadex G-50 followed by ion-exchange chromatography on DEAE cellulose and then affinity chromatography on Sepharose 4B. The lectins were found to be homogeneous justified by polyacrylamide disc gel electrophoresis. The molecular weights of the lectins CSL-1, CSL-2 and CSL-3, determined by gel filtration on Sephadex G-75 column were 37,000, 42,400 and 46,000, and by SDS gel electrophoresis were 37500, 42000 and 46500, respectively. The lectins agglutinated rat red blood cells and the agglutination was inhibited specifically by galactose and galactose containing saccharide. The neutral sugar contents of the lectins, CSL-1, CSL-2 and CSL-3 were estimated to be 3.5, 3.1 and 2.0 % respectively. The sugar composition of the lectins was found to be galactose for CSL-1, galactose and glucose for CSL-2, and galactose and mannose for CSL-3. The lectins displayed strong cytotoxic effect in brine shrimp lethality bioassay and among the lectins purified, CSL-2 was found to be highly toxic followed by CSL-1 and then CSL-3. The purified lectins in aqueous solution gave absorption maxima around 274-278 nm and minima around 243-248 nm.

Biological activities of the lectins CSL-1, CSL-2 and CSL-3, were investigated after various physico-chemical treatments. Biological activities were highly affected with the changes of pH and temperature, and the lectins exhibited maximum hemagglutinating activities around pH 7.2 to 7.5 and at temperature 20^0 to 35 ^{0}C. Biological activities of the lectins were abolished sequentially with the increase in concentration of acetic acid and denaturant solutions such as urea and guanidine-HCl.

The solvent extraction of *Cassia fistula* seed collected from three districts viz; Rajshahi, Rangpur and Dhaka yielded about 3 % oil in average. The important physico-chemical characteristics of the oils in the sample of Rajshahi, Rangpur and Dhaka studied by conventional methods, were specific gravity (0.9259-0.9265), refractive index (1.4562-1.4753), acid value (2.77-3.41), FFA (1.39-1.71 %), saponification value (186.37-188.40), saponification equivalents (297.77-301.01), unsaponifiable matters (5.00-5.50 %), iodine value (95.53-96.83), ester value (183.79-184.99), acetyl value (18.81-21.21), Reichert-Meissl value (0.78-0.89) and peroxide value (2.55-2.90). The oils were fractionated into mono-, di- and triglycerides by silicic acid column chromatography. The triglycerides varied from 89.17 to 91.01 %, diglycerides from 2.51 to 3.32 % and monoglycerides from 0.96 to 0.98 %. Fractionation of lipids extracted from the seeds into three major lipid groups neutral lipids, glycolipids and phospholipids was carried out by silicic acid column chromatography. Neutral lipids, glycolipids and phospholipids amounted to 86.10-89.79 %, 3.02-4.54 % and 1.82-2.70 % respectively. Saturated and unsaturated fatty acids present in the oils were separated and varied from 25.75 to 26.40 % and 66.71 to 68.11 % respectively depending on the areas from where the seeds were collected. The major fatty acids found in the oil were linoleic acid (42.42 %), oleic acid (29.62 %), stearic acid (14.33 %) and palmitic acid (11.41 %). In addition to the above, caprylic acid (0.76 %) and myristic acid (1.44 %) were also present in minor amount.

The powdered materials of stem bark, leaves, pods and seeds of *Cassia fistula* were subjected to extract separately with petroleum ether, ethyl acetate and methanol. Three compounds were isolated from crude ethyl acetate extract of *Cassia fistula* stem bark and were finally identified as β-Sitosterol (CBC-1), Bet-20 (29)-en-3-ol-28-oic acid (CBC-2) and lupeol (CBC-3) on the basis of physico-chemical and spectral evidences. All the crude extracts obtained from stem bark, leaves, pods and seeds as well as two pure compounds CBC-2 and CBC-3 isolated from the stem bark were tested for their antibacterial and antifungal activities against 14 pathogenic bacteria and 6 fungi respectively. Three lectins CSL-1,

CSL-2 & CSL-3, purified from the seeds were also screened for their only antibacterial activities against 14 pathogenic bacteria using 20µg/disc. Among them, CSL-3 was found to be more active against most of the bacteria tested. The crude extracts (400 µg/disc) and pure compounds (200 µg/disc) showed mild to strong activity against most of the bacteria and comparatively low activity against a few of the total fungi tested. The minimum inhibitory concentrations (MICs) of CBC-2 against *Bacillus subtilis* and *Shigella sonnei* were found to be same i. e., 32 µg/ml as well as of CBC-3 against *Bacillus subtilis* and *Shigella dysenteriae* were estimated to be 64 µg/ml and 32 µg/ml respectively when tested in nutrient broth medium. In bioassay of brine shrimp lethality, the values of LC_{50} of petroleum ether, ethyl acetate and methanol extracts were 13.85, 11.00 and 17.64 µg/ml for stem bark, 13.96, 23.26 and 18.05 µg/ml for leaves as well as 16.10, 9.99 and 19.49 for pods respectively. LC_{50} values of isolated pure compounds, CBC-2 and CBC-3, were found to be 14.01 and 10.39 µg/ml respectively.

The biochemical compositions of *Cassia fistula* seeds and leaves were extensively investigated. The leaves contained about 186.09 mg % total chlorophyll, 137 mg % total phenol, 65.96 gm % moisture, 2.75 gm % ash, 1.88 gm % lipid, 5.87 gm % total protein, 2.91 gm % water soluble protein, 9.97 gm % crude fiber, 13.57 gm % total carbohydrate and 94.68 Kcal/100 gm energy value. On the other hand, seeds contained about 7.65 gm % moisture, 4.63 gm % ash, 3.85 gm % lipid, 22.91 gm % total protein, 11.56 gm % water soluble protein, 7.64 gm % crude fiber, 53.32 gm % total carbohydrate and 339.57 Kcal/100 gm energy value.

List of Table

Table No. Page No.

Table-2.1: Extraction of crude proteins from *Cassia fistula* seeds in different extracting solvents. 18

Table-2.2: Purification of c*assia fistula* seed proteins 31

Table-2.3 Hemagglutinating activities of *Cassia fistula* seed proteins with 4% red blood cells (RBC) from albino rat. 35

Table-2.4: Hemagglutination Inhibition assay of *Cassia fistula* seed lectins by different sugars. 37

Table-2.5: Optical density (O. D.) and concentration relationship of the proteins. 38

Table-2.6: Effect of *Cassia fistula* seed lectins on brine shrimp lethality bioassay 40

Table-3.1: Hemagglutinating activities of *Cassia fistula* seed lectins at different pH values. 47

Table-3.2: Effect of temperature on Hemagglutinating activities of *Cassia fistula* seed lectins. 48

Table-3.3: Effect of acetic acid at different concentrations on Hemagglutinating activities of *Cassia fistula* seed lectins. 49

Table-3.4: Effect of urea at different concentrations on Hemagglutinating activities of *Cassia fistula* seed lectins. 50

Table-3.5: Effect of Guanidine-HCl at different concentrations on hemagglutinating activities of *Cassia fistula* seed lectins. 51

Table-4.1: Physical and chemical characteristics of *Cassia fistula* seed oil. 65

Table-4.2: Glyceride composition of *Cassia fistula* seed oil (weight %). 67

Table-4.3: Lipid composition of *Cassia fistula* seed oil (weight %). 67

Table-4.4: Percentage of saturated and unsaturated fatty acids 68

Table-4.5: The fatty acid composition of *Cassia fistula* seed oil (weight %) 69

Table-5.1: Column chromatography of crude ethyl acetate extract over silica gel (70-230 mesh). 75

Table-5.2: Developing solvent systems with R_f values of the pure compounds. 77

Table-5.3: Solubility behavior of the pure compounds. 77

Table-5.4: ^{1}H-NMR (500 MHz) spectral data of compound CBC-1. 79

Table-5.5: ^{13}C-NMR (125 MHz) spectral data of compound CBC-1. 79

Table-5.6: ^{1}H-NMR (500 MHz) spectral data of compound CBC-2. 80

Table-5.7: ^{1}H-NMR (400 MHz) spectral data of compound CBC-3. 81

Table-5.8: ^{13}C-NMR (100 MHz) spectral data of compound CBC-3. 82

Table-6.1: Percentage of yield of different solvent extracts of the plant *Cassia fistula*. 100

Table-6.2: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* stem bark and standard antibiotic Kanamycin against bacterial strains. 103

Table-6.3: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* leaves and standard antibiotic Kanamycin against bacterial strains. 104

Table-6.4: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* pod and standard antibiotic Kanamycin against bacterial strains. 105

Table-6.5: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* seed and standard antibiotic Kanamycin against bacterial strains. 106

Table-6.6: Zone of inhibition exhibited by the compounds CBC-2 & CBC-3 purified from *Cassia fistula* stem bark as well as lectins CSL-1, CSL-2 & CSL-3 purified from *Cassia fistula* seeds and standard antibiotic Kanamycin against bacterial strains. 107

Table-6.7: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* stem bark and blank disc against fungal strains. 110

Table-6.8: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* leaves and blank disc against fungal strains. 110

Table-6.9: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* pod and blank disc against fungal strains. 111

Table-6.10: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* seeds and blank disc against fungal strains. 111

Table-6.11: Zone of inhibition exhibited by pure compound-CBC-2 & CBC-3, isolated from *Cassia fistula* stem bark and blank disc against fungal strains. 112

Table-6.12: Minimum inhibitory concentrations (MIC) of the pure compound CBC-2 isolated from *Cassia fistula* stem bark against gram-positive *Bacillus subtilis* and gram-negative *Shigella sonnei.* 115

Table-6.13: Minimum inhibitory concentrations (MIC) of the pure compound CBC-3 isolated from *Cassia fistula* stem bark against gram-positive *Bacillus subtilis* and gram-negative *Shigella dysenteriae.* 116

Table-6.14: Effect of crude extracts of *Cassia fistula* stem bark on brine shrimp lethality bioassay. 118

Table-6.15: Effect of crude extracts of *Cassia fistula* leaves on brine shrimp lethality bioassay. 120

Table-6.16: Effect of crude extracts of *Cassia fistula* pod on brine shrimp lethality bioassay 122

Table-6.17: Effect of Pure compounds isolated from *Cassia fistula* stem bark on brine shrimp lethality bioassay. 124

Table-7.1: pH, TTA, Chlorophyll and Total Phenol Contents of the *Cassia fistula* leaves 131

Table-7.2: Major proximate nutritional compositions of seeds and leaves of *Cassia fistula*. 135

List of Figure

Figure No. Page No.

Figure-2.1: Gel filtration of 100 % $(NH_4)_2SO_4$ saturated crude protein extract on Sephadex G-50 column. 24

Figure-2.2: Ion exchange chromatography of fraction, F-2 on DEAE-cellulose (linear elution) 25

Figure-2.3: Ion exchange chromatography of fraction F-2 on DEAE-cellulose. (stepwise elution) 26

Figure-2.4: Affinity chromatography of fractions F-2b (6.08 mg), F-2c (10.38 mg) and F-2d (6.29 mg) obtained from DEAE-cellulose column, on Sepharose 4B. 27

Figure-2.5: Polyacrylamide disc gel electrophoretic patterns of the purified proteins at room temperature on 7.5 % gel. 2.5

Figure-2.6: Flow diagram for purification of proteins from *cassia fistula* seeds. 29

Figure-2.7: Standard curve for determination of molecular weight by gel filtration on Sephadex G-75. 33

Figure-2.8: Standard curve for determination of molecular weight by SDS- polyacrylamide gel electrophoresis. 34

Figure-2.9: Agglutination of albino rat red blood cells by CSL-1 36

Figure-2.10: Agglutination of albino rat red blood cells by CSL-2 36

Figure-2.11: Agglutination of albino rat red blood cells by CSL-3 36

Figure-2.12: Determination of LC_{50} of *Cassia fistula* seed lectins 39

Figure-2.13: Ultraviolet absorption spectra of CSL-1, CSL-2 and CSL-3 lectins in aqueous solution. 41

Figure-5.1: Extraction of plant materials using solvents of various polarities. 73

Figure-5.2: ^{1}H-NMR spectrum of compound CBC-1 85

Figure-5.3: ^{13}C-NMR spectrum of compound CBC-1 86

Figure-5.4: IR spectrum of compound CBC-2 89

Figure-5.5: ^{1}H-NMR spectrum of compound CBC-2 90

Figure-5.6: IR spectrum of compound CBC-3 93

Figure-5.7: ^{1}H-NMR spectrum of compound CBC-3 94

Figure-5.8: ^{13}C-NMR spectrum of compound CBC-3 95

Figure-5.9: Mass spectrum of compound CBC-3 96

Figure-6.1: Determination of LC_{50} of crude extracts of *Cassia fistula* stem bark 119

Figure-6.2: Determination of LC_{50} of crude extracts of *Cassia fistula* leaves 121

Figure-6.3: Determination of LC_{50} of crude extracts of *Cassia fistula* pod 123

Figure-6.4: Determination of LC_{50} of pure compounds CBC-2 and CBC-3 isolated from *Cassia fistula* stem bark. 125

List of Abbreviation

BSA	Bovine serum albumin
DEAE-cellulose	Diethylaminoethyl cellulose
PBS	Phosphate buffer saline
UV	Ultraviolet
SDS	Sodium dodecyl sulfate
OD	Optical density
MHD	Minimum hemagglutination dose
$CDCl_3$	Deuteriochloroform
NMR	Nuclear Magnetic Resonance
IR	Infra-red
TLC	Thin layer chromatography
PTLC	Preparative thin layer chromatography
TMS	Tetramethylsilane
δ	Chemical shift in ppm
s	Singlet
d	Doublet
dd	Double doublet
R_f	Retardation factor
FFA	Free fatty acid

Chapter-1

General Introduction and Literature Review

General Introduction and Literature Review

1.1 General

Man, on the average, is likely to consider himself as a being apart from the rest of the organic world, enabled by reason of his superior intellect to lead a self-sufficient and independent existence. He losses sight of the fact, or is ignorant of it, that he is absolutely dependent on other organisms for his very life, and his material happiness as well. Although various animal and mineral products contribute to his welfare, it is the plant kingdom that is most essential to man's well being.

Man's dependence on plants for the essential of his existence has been of paramount importance in his life since the human race began. Primitive man probably had few needs other than food and a little shelter. Civilization, however, has brought with it an ever-increasing complexity, and has increased man's requirements to an amazing degree. The man of today is no longer content merely to exist, with food and shelter as his only wants. He desires other commodities as well, and raw materials that can be converted into the many useful articles and products which contribute to his enjoyment of life, and which incidentally, increase his debt to plants.

The three great necessities of life --food, clothing and shelter and a host of other useful products are supplied in great part by plants. An adequate food supply is, and always has been, man's most outstanding need. In the last analysis all his food comes from plants. To be sure he may eat the flesh of animals, but these lower animals are just as dependent on plants as man himself is, and they are equally

unable to manufacture any of their food from raw materials. Clothing and shelter, the other prime necessities of life, are derived in great part from plant fiber and from wood.

The use of plants and plant products is very old. The earliest records of therapeutic use of plants can be seen in the ancient literatures of the Chinese, the Hindus, the Romans, the Greek and the Egyptians. Among the earliest people working on medicinal properties and uses of plants, mention must be made of Theophrastus (370-285 B.C.), a student of Plato, the great Greek physician, Dioscorides, the famous Roman physician pliny (Core, 1962), and the Chinese Emperor and physician Shen Nung (300 B.C) . Shen Nung described the antifebrile effect of the Chinese drug change, now shown to contain antimalarial alkaloids. Mahuang recommended by Shen Nung (Burger Alfred, 1960) as stimulant and diaphoretic, was found, almost 5000 years later, to contain ephedrine.

About a generation ago, the use of plants and herbs as remedial agents was greatly discredited. The late Sir Thomas Lauder Brunton drew an analogy between the weapons and tools employed in art or warfare, and implementation in the treatment of disease of man in different ages. We may recognize four stages in the development of implementation in the treatment of disease. In the first stage crude drugs were employed, prepared in the roughest manner, such as powdered Cinchona or metallic antimony. In the next stage, these were converted into more active and more manageable forms, such as extracts or solutions, watery or alcoholic. In the third stage, the pure active principles, separated from the crude drugs, were employed, e.g. morphine and quinine. In the fourth stage, instead of attempting to extract our medicines from the natural products in which they are contained, we seek to make for ourselves such substances as shall possess the particular action we desire (Kirtikar and Basu, 1987).

The knowledge of medicinal plants must have been accumulated in many countries. It is greatly to the credit of the people of India that they were acquainted with a far larger number of medicinal plants than the natives of any other country on the face of the earth. The vegetable materia medica of the Greek, Romans, Egyptians, Jews, Babylonians, persians, Chinese and Arabs do not display such an extensive knowledge of medicinal plants and drugs as does any of the authoritative medical works of the Hindus. The knowledge of herbs possessed by the aborigines of America, Australia or Africa, is also not very great (Kirtikar and Basu, 1987).

Over 7000 species of plants found in different Eco-systems are possibly in use for medicinal purposes in India by different communications. This forms more than one third of the known higher plant species of India. In the area of commercial use of medicinal plants it is generally accepted that nearly 95 % of the medicinal plants, in use for manufacturing various indigenous formulations, are being obtained from the wild (Ved, 1994).

Bangladesh is unique in possessing one of the largest deltaic plains of the world with extensive inland depressions. Out of total area of 1,42,487 sq. km. of the country, almost 1/8 th is occupied by littoral forest in the south while the rest is comprised of more than 1,24,352 sq. km. of flood plains excepting the hills in the north-east and south-east. The fertile plains, forests, hills and low lying plains (contain aquatic flora) of the country are occupied by enormous plant resources which provide food and medicine for rural population (Khan and Halim, 1987). The people of Bangladesh use some of these plants for the treatment of various diseases. These plants generally owe their virtues as medicinal agents to certain characteristic alkaloids and principles present in them. Isolation of active principles from indigenous plants may constitute a great importance in pharmacy.

The chemical analysis would also help us in determining the action of active principles in health and disease. Unfortunately no serious systematic survey of the available plant resources has yet been made in Bangladesh. In such a situation, it is worthwhile to study the medicinal plants of Bangladesh origin.

1.2 Leguminosae, A Plant Family

Leguminosae is a large plant family consisting of herbs, shrubs or trees, leaves stipulate and usually alternate, pinnate or digitale or simple, often stipellate, sometimes with the rachis ending in a tendril. Inflorescence axially leaf-opposed or terminal, usually simply racemes or panicled; bracts and bractcoles usually both present. Flowers usually irregular, hermaphrodite, rarely regular or polygamous. Sepals 5, combined or free, often unequal, sometimes combined into two lips petals 5, rarely fewer by arrest, usually free and unequal, Stamens normally 10, perigynous or almost hypogynous, rarely fewer by arrest or indefinite; filaments free or variously combined. Ovary free; style simple, cylindrical, usually declinate; stigma capitate, terminal or oblique. Ovules one or more on the vental suture. Fruit usually dry, a pod splitting open along both sutures, sometimes continuous and indehiscent, at other separeting into 1-seeded joints. Seeds usually exalbuminous; cotyledons foliaceous or amygdaloid, with a straight or inflexed accumbent radicle (Hooker, 1879).

1.3 Information About the Plant , *Cassia fistula*

The Genus *Cassia*

A large and predominantly tropical genus of about 580 species of herbs, shrubs and trees, with about 20 representations including ***Fistula*** are found in indo-Pak-Bangladesh subcontinent. Leaves simple, abruptly pinnate. Flowers usually large and showy, in axillary racemes and terminal panicles. Calyx-tube very short; sepals broad or narrow, imbricated petals 5, imbricated, subequal, usually broad.

Stamens normally 10, but rarely all perfect, 3-5 being often reduced to staminodia or altogether absent. Ovary sessile or stalked, many-ovulated; style incurved, stigma terminal. Pod very variable, terete or flat, usually septate, the albuminous seeds flattened, sometimes parallel with the valves, sometimes with the septa, dry, dehiscent or indehiscent (C.S.I.R., 1950).

Botanical Information About *Cassia fistula* (Salar and Alam, 1996; Blatter and Millard, 1954)

Botanical name : *Cassia fistula* Linn

Family : Leguminosae.

Subfamily : Caesalpinoideae.

Vernacular name :

Bengal : Amultas, Bandarlati, Sonali, Sondala, Sondali, Sudali.

English : Cassia, Drumstic, Golden shower, Indian laburnum.

Hindi : Amaltas, Bandarlauri, Girmalah, Hamaltas, Khyar, aikassi.

Arabic : Bukbur, Chiar-schambar, Katha-ul-hind, Khiyar hambur.

Assam: Sonaru, Sunaru

Panjab : Ali, Alash, Amaltas, Kaniar, Karangal, Kiar.

Burma : Gnookye, Gnooshway, Ngu.

Chinese : A Po Le, Koui Hoa Ts'in, Tch'ang Ko Chou.

Indo-China : Bo cap muoc, brai xiem, Krete, Sach phle.

Egypt : Chiar schambar.

Greek: Glykokalamon, Kassiamelaina, Melaina kassia, Syrina.

Hawaii: Golden shower

Sanskrit: Mirjubaha, Nurue, Nuruic

Sinhalese: Ahalla, Ahilla, Ehela

A medium sized tree. Leaves paripinnate, 20-40 cm long; leaflets 4-8 pairs, opposite, 5-15 x 3-8cm, ovate or elliptic, dark-green and shining above,

undersurface silvery pubescent. Flowers yellow in axillary, 20-60 cm long drooping racemes. Fruit a pod, 30-60 cm long, 2-3 cm across, cylindric, indehiscent, smooth between the seeds. Flowering time: March-June; Fruiting time: cold season.

Medicinal properties and Uses

In Hindu medicine, the pulp is used as a cathartic; and the root is also described as a laxative, useful in fever, heart disease, retained excretions, biliousness, etc. In the Makhzan-El-Adwiya, the pulp is described as lenitive, useful for relieving thoracic obstructions and heat of blood, and is a safe aperient for children and women. Externally, it is said to be a good application for gout, rheumatism, etc. The flowers are made into a confection known as *Gul-kand* and viewed as a febrifuge. From 5 to 7 of the powdered seeds are prescribed as an emetic and the shell of the pod, rubbed down with saffron, sugar and rosewater, in difficult parturition. In the Konkan, the juice of the young leaves is used to cure ringworm and allay the irritation caused by the application of the marking-nut juice.

The root is given as a tonic and febrifuge. It has been found to act as a strong purgative. A poultice made of the leaves is said to relieve the chilblains, which are common in Upper Sind. It has been beneficially used in facial paralysis and rheumatism when rubbed into the affected parts. Internally, it is given as a derivative in paralysis and brain affections. The pulp of the fruit is in common use as a purgative in the South of Europe; but is not often employed in England, except in the form of the lenitive electuary, of which it is an ingredient (Blatter and Millard, 1954).

1.4 Literature Survey on *Cassia fistula*

Luximon-Ramma *et. al* (2002) determined the total phenolic, proanthocyanidin, and flavonoid contents, and the antioxidant activities, of fresh vegetative and

reproductive organs of *Cassia fistula* harvested at different stages of growth using the Trolox equivalent antioxidant capacity (TEAC) and ferric-reducing antioxidant power (FRAP) assays.

Kuo ***et. al.*** **(2002)** isolated and identified four new compounds, 5-(2-hydroxyphenoxymethyl) furfural, (2'S)-7-hydroxy-5- hydroxymethyl-2-(2'-hydroxypropyl) chromone, benzyl 2-hydroxy-3,6-dimethoxybenzoate, and benzyl 2 β-O-D-glucopyranosyl-3,6-dimethoxybenzoate, together with four known compounds, 5-hydroxymethylfurfural, (2'S)-7-hydroxy-2-(2'-hydroxypropyl)-5-methylchromone, and two oxyanthraquinones, chrysophenol and chrysophanein from the seeds of *Cassia fistula*.

Bhakta ***et. al.*** **(2001)** investigated hepatoprotective activity of the n-heptane extract of *Cassia fistula* leaves by inducing hepatotoxicity with paracetamol in rats.

Ching-Kuo Leea ***et. al.*** **(2001)** isolated and identified Twenty-seven compounds including eight long-chain hydrocarbons, 1-hexacosanol, 1-octacosanol, palmitic acid, stearic acid, oleic acid, linoleic acid, heptacosyl eicosanate, glyceryl-1-tetraeicosanoate; three sterols, β-sitosterol, stigmasterol, β-sitosteryl-3-O-D-glucopyranoside, one triterpene, eight anthraquinones, chrysophanol, emodin, physcion, citreorosein, rhein, rhein methyl ester, ziganein, 1,4,5-trihydroxyanthraquinone, two coumarins, isoscopoletin, scopoletin, two chromones, 2,5-dimethyl-7-hydroxychromone, 2,5-dimethyl-7-methoxychromone, three aromatic compounds, isovanillic acid, vanillic acid, and 2,4-dihydroxybenzaldehyde from the bark of *Cassia fistula*.

Akiremi ***et. al.*** **(2000)** examined laxative properties of the pods of *Cassia fistula* L. and *C. podocarpa* macro- and microscopially. The pods can be distinguished in

powder form by the presence of uniseriate trichomes in the *C. podocarpa,* which are absent in *C. fistula.* Chemical evaluation showed that both pods contain free and combined (O- and C- glycosides) anthraquinones, while biological evaluation of the infusions of the pods of the two *Cassia* species showed that there is a significant difference in the amounts of wet faces produced, with *C. fistula* showing a higher activity than *C. podocarpa* at the same dose levels when compared with Senna (*Cassia acutifolia* Delile) under the same experimental conditions. The biological Senna equivalent and percentage Senna activity for *C. fistula* and *C. podocarpa* are 1.13, 113% and 0.70, 70% respectively at 500 mg/kg. Both *Cassia* species pods can substitute for the official Senna.

Wijaya *et. al.* (2000) extracted a novel trypsin inhibitor from the seeds of *Cassia fistula* by a process successively soaking seeds in water, extraction of the seeds in methanol, and extraction of the cell wall material at high ionic strength. The protease inhibitor (PI) was subsequently purified by chromatography on carboxymethylcellulose, gel filtration and reversed phase HPLC (RP-HPLC).

Gupta *et. al.* (2000) studied the effects of methanolic extract (ME) of *Cassia fistula* seed on the growth of ehrlich ascites carcinoma (EAC) and on the life span of tumor bearing mice. ME treatment showed an increase of life span, and a decrease in the tumor volume and viable tumor cell count in the EAC tumor hosts. Cytological studies have revealed a reduction in the mitotic activity, and the appearance of membrane blebbing and intracytoplasmic vacuoles in the treated tumor cells. Improvement in the hematological parameters following ME treatment, like hemoglobin content, red blood cell count and bone marrow cell count of the tumor bearing mice has also been observed. The results of the present study suggest that ME of *C. fistula* seed has an antitumor activity.

Raja *et. al.* (2000) studied the effect of solvent residues of *Vitex negundo* L. and *Cassia fistula* L. leaves (0.5 and 1%) on egg laying and adult emergence of Callosobruchus maculatus Fab. and on percentage of larval parasitism by Dinarmus vagabundus (Timberlake).

Yadav and Jain (1999) administrated orally the aqueous extract of seeds of *Cassia fistula* to mated female rats from day 1-5 of pregnancy at the doses of 100 and 200 mg/kg body weight, and 57.14% and 71.43% prevention of pregnancy, respectively were observed whereas 100% pregnancy inhibition was noted at 500 mg/kg bw.

Abo *et. al* (1999) estimated colorimetrically the anthraquinone content, antimicrobial and laxative effects of leaves and pods of *Cassia fistula* Linn., *C. specatabilis* DC and *C podocarpa* because of the popular uses of these species by herbalists in Ibadan. The pods of the *Cassia* species exhibited potent antifungal activity than the leaf samples. Pods of *C. fistula* showed significant antibacterial activity when compared to that of ampicillin. This study justifies the use of the *Cassia* species in traditional medicine.

Elujoba *et. al.* (1999) carried out detailed biological evaluation for laxative properties using established literature methods with white albino rats on the infusions of the pods of the following *Cassia* species, growing in Nigeria: *Cassia alata* L., *C. fistula, C. hirsuta* L., *C. occidentalis* L., *C. podocarpa* Guill. Et Perr., *C. siamea* Lam. and *C. sieberiana* L. (Family Caesalpinoidae) with *Cassia acutifolia* Del. (Senna) pod tablet and leaf as reference standards.

Mukhopadhyay *et. al.* (1998) investigated anthraquinone glycosides of *Cassia angustifolia* and *C. fistula* for their ability to induce a clastogenic effect on the bone marrow cells of Swiss albino mice.

Perumal Samy *et. al.* (1998) assayed a total of 34 plant species belonging to 18 different families for antibacterial activity against *Escherichia coli, Klebsiella aerogenes, Proteus vulgaris,* and *Pseudomonas aerogenes* (gram-negative bacteria) at 1000-5000 ppm using the disc diffusion method. Of these 16 plants showed activity; among them *Cassia fistula, Terminalia arjuna* and *Vitex negundo* showed significant antibacterial activity against the tested bacteria. Our findings confirm the traditional therapeutic claims for these herbs.

Rani and Kalidhar (1998) isolated a novel anthraquinone, 3-formyl-1-hydroxy-8-methoxy-anthraquinone from the pod of *C. fistula.*

Das and Jogi (1998) isolated an anthraquinone derivative 1,8-Dihydroxy-6-methoxy-3,7-dimethyl anthraquinone from the root of *C. fistula.*

Misra *et. al.* (1997) isolated a new diterpene, 3 β-17-norpimar-8 (9)-en-15-one from the pod of *C. fistula.*

Joshi (1997) published a paper which deals with the effect of urban air pollution on *Cassia fistula* and *Cassia siamea* at Indore city. A reducing trend in total chlorophyll and ascorbic acid wash recorded during winter in both the species. In case of *C. siamea,* the values were higher except textile mill area, for both the species. On overall basis *C. fistula* appeared more sensitive to urban air pollution than *C. siamea.*

Misra *et. al.* (1996) isolated two new compounds with antibacterial activity ; 5-nonatetracontanone and 2-hentriacontanone from the hexane fraction of the pod of *C. fistula.*

Barthakur *et. al.* (1995) analyzed the edible fruit tissue of Indian laburnum fruit (*Cassia fistula* L.), a member of the leguminosae family for certain organic

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compounds and mineral nutrients. Of the nine macro- and micronutrients studied, K was the most highly concentrated such that 100% of the US Recommended Dietary Allowances (RDA) for adults could be met by the consumption of about 100 g of the fresh fruit. Na contents in pulp and seeds are relatively low. Ca content at 827 mg per 100 g of dry matter is one of the highest of any fruits, and could contribute towards the RDA requirement of 800 mg of Ca for adults per day. The fruit is a good source of Fe and Mn, and their concentrations are considerably higher than those found in apple, apricot, peach, pear, and orange. Aspartic acid, glutamic acid, and lysine constituted 15.3, 13.0, and 7.8% of the total amino acids respectively in the pulp. In the seeds the same amino acids constituted, 16.6, 19.5, and 6.6%. The relatively high energy content of the fruit at 18 kJ/g could enhance the daily energy requirement of people in need of adequate caloric intake. The results of the present study demonstrate that the Indian laburnum fruit could be a source of some important nutrients and energy for humans.

El-Saadany *et. al.* (1991) investigated the hypocholesterolaemic effect of *Cassia fistula* using hypercholesterolaemic male albino rats. Hypercholesterolaemia was induced by feeding on a mixture of cholesterol plus cholic acid for a 12 weeks period.

Daulatabad *et. al* (1987) analyzed the seed oils of *C. fistula* and *C. renigera.* The *C. fistula* and *C. renigera* seed oils contained 6.1 and 4.4 % vernolic acid, respectively. The seed oil of *C. fistula* contained malvalic (1.5%) and sterculic acids (2.0%) as its cyclopropenoid fatty acids.

El-Sayyad *et. al.* (1985) studied phytochemically the fruits of certain Cassia specis cultivated in Egypt. The pericarps of *C. fistula, C. javanica, C. siamea* and *C. didymobotrya* contained flavonoids, anthraquinones, chromones, alkaloids,

sterols and (or) triterpenes together with hydrocarbons and alcohol. The semi-drying seed oils contained too much FFA, waxes and hydrocarbons to be used for food.

Vasi and Kalintha (1980) examined chemically the fruit pulp of *C. fistula* Linn. The fruit pulp of *C. fistula* is rich in proteins (19.94 %) and carbohydrate (26.30 %).

Gupta *et. al.* (1975) carried out a research work on chemical composition and *in vitro* nutrient digestibility of some of the tree (including *C. fistula)* leaves. The dry matter of leaves of 23 different species of trees ranged (percent) 17.2-49.3, crude protein 9.2-21.0, neutral detergent fiber 18.5-76.0, total mineral 9.25-21.5, Ca 1.12-4.0, P 0.10-0.60, and SiO_2 0.3-5.2. Leaves of most species could be used for animal feed.

1.5 Rationale of the Present Work

Cassia fistula L., a semi-wild Indian Laburnum, is frequently planted on city roads and avenues in almost all the districts of Bangladesh as a handsome ornamental tree for its beautiful bunches of yellow flowers and also used in traditional medicine for several indications from the time immemorial. The seed of *Cassia fistula has been* attracted much attention since it contains about 24 % protein as reported by Roskoski *et. al.* (1980) which is higher than that contained in most of the plant sources and may be considered as an important source of lectins. Lectins are sugar-binding proteins that agglutinate cells and / or precipitate glycoconjugate molecules with a carbohydrate portion like polysaccharide, glycoproteins, glycolipids and other. Lectins play a key role in the control of various normal and pathological processes in living organisms. Research in the field of lectins has been going on in many research laboratories of the world. So far more than hundred lectins have been purified and characterized but their detailed molecular mechanism has not yet been understood. Since lectins isolated from different sources have unique specificities and are useful reagents for glycoconjugate

separation as well as possess many biological properties, so many laboratories in different countries have been continuing research in the field of lectins. No such information is available about the nature and types of proteins present in the seed of *Cassia fistula* while this plan was undertaken for working. Hence systematic studies have been undertaken on the isolation, purification and characterization of proteins from the seed of *Cassia fistula* grown in the climatic conditions of Bangladesh adopting all the modern methodology and techniques developed in the field of protein technology.

In addition to large amount of protein, the *Cassia fistula* seed contains about 3 % oil, possessing a honey-like odour. Fats and oils are very important indigenous raw materials for many purposes specially for edible and non-edible purposes. Lot of investigations were carried out on vegetable fats and oils but there are also many plants yet to be studied. Till now no detailed studies were done on the fatty acid composition and physico-chemical properties of the *Cassia fistula* seed oil. The physico-chemical properties of fats or oils are directly related to their glyceride composition and chemical constitution. Consequently knowledge of these compositional factors is important in connection with research aimed at improvement of fat and fat products for specific use. In view of the above facts, I became interested to investigate the seed oil of *Cassia fistula*.

Again a survey of literature reveals that the plant *Cassia fistula* is an important source of biologically active compounds, which may exhibit physiological functions after administration. These physiologically active principles of this plant are of great importance from the medicinal point of view. Some phytochemical and microbiological works have been carried out, but no systematic research work on this plant was found anywhere in the literature as yet, although this plant is very important source of secondary metabolites. So our attention was concentrated to carry out a complete phytochemical and antimicrobial studies on the plant

Cassia fistula to isolate and characterize the constituents interested both for phytochemically and microbiologically. At the end, our research work has extensively been focussed on the biochemical analysis of various portion of the abovesaid plant.

1.6 Present Study Protocol

The present study envisages into the chemical, microbiological and toxicological investigations on *Cassia fistula* which includes :

Chepter-2: Isolation, purification and characterization of proteins from the seeds of *Cassia fistula.*

Chepter-3: Effect of physical and chemical agents on the Hemagglutinating activities of proteins purified from the *Cassia fistula* seed.

Chepter-4: Physico-chemical investigations on *Cassia fistula* seed oil.

Chepter-5: Isolation, purification and characterization of compounds from the stem bark of *Cassia fistula.*

Chepter-6: Microbiological and toxicological investigations of pure proteins, crude extracts and pure compounds obtained from *Cassia fistula.*

Chepter-7: Biochemical analysis of seeds and leaves of *Cassia fistula.*

Chapter-2

Purification and Characterization of Proteins from *Cassia fistula* Seed

Purification and Characterization of Proteins from *Cassia fistula* Seed

2.1 Introduction

Although animal kingdom is main sources of protein, but plant kingdom also possesses a lot of protein. Green leaves, barks, roots, stems etc. contain small amount of protein, while seeds are main sources of protein in plants. Some plant proteins are toxic and some are non-toxic. Most of the plant seed contains glycoprotein. Glycoproteins are protein polysaccharide compounds. They contain in general, 1-3% carbohydrate but there are exceptional cases in which the carbohydrate content may be about 10-12 % (Lis and Sharon, 1989). The sugar moieties generally found are mannose, glucosamine, xylose, glucose, galactose etc. Among them mannose and glucosamine are predominates.

Lectins, a class of proteins that bind sugar specifically and reversibly and that agglutinate cells, are widely distributed in nature, being found in animals, insects, plants and microorganism (Lis and Sharon, 1990). The term lectin was proposed by Boyd (1970) because of their unique carbohydrate binding properties. Plant lectins are the first and still the largest and best-characterized group. It becomes apparent that many of the lectins could be grouped into families with sequence homologies and common structural properties. The largest and best-characterized family is that of the Leguminoseae lectin. Two small families, also of plant lectins, are those from *Gramineae* (cereals) lectin and *Solanaceae* (e.g. potato and tomato) lectin (Lis and Sharon,1990). The main source of lectin is mature seeds. Small amount of lectins are present in other tissues such as leaves, bark, and roots (Chrispeels and Tague,1990).

Most plant tissues contain one lectin, but in some cases two or more lectins that differ in their sugar specificities and other properties are present. They are classified into a small number of specificity groups (mannose, galactose, N-acetylglucosamine, N-acetylgalactosamine, and N-acetylneuraminic acid) according to the monosaccharide that is the most effective inhibitor of the agglutination of erythrocytes or precipitation of carbohydrate containing polymers by the lectin.

Lectins are currently attracting much interest, primarily because they serve as invaluable tools in diverse areas of bio-medical research. Because of their unique carbohydrate binding properties, lectins are useful for the separation and characterization of glycoproteins and glycolipids, in studies of glycolipids; following changes that occur on cell surfaces during physiological and pathological processes, from cell differentiation to cancer; histochemical studies of cells and tissues; tracing neuronal pathways; typing blood cell and bacteria; and for fractionation of lymphocytes and of bone marrow cells for bone marrow transplantation. They are also used to stimulate lymphocytes to assess the immune state of patients and for chromosome analysis in human cytogenetics, as well as for the production of cytokinases. In addition, lectins are excellent models for examining the molecular basis of specific reaction that occur between proteins and other types of molecules, both of low or high molecular weight, such as the binding of antigens to antibodies, of substrates to enzymes, of drugs to proteins and of hormones and growth factors to cells.

The present study was, therefore, undertaken with the objective of purifying the proteins from the *Cassia fistula* seeds in the biologically active form. Furthermore, these proteins were also characterized, with respect to the molecular weight, neutral sugar composition, cytoagglutination, toxicity etc.

2.2 Materials and Methods

2.2.1 Chemicals

Sephadex G-50, Sephadex G-75, and Sepharose-4B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose was purchased from Sigma Chemical Co., USA. Molecular weight markers were the products of Fluka Biochemica, Switzerland. All the other reagents used were of analytical grade. Unless otherwise specified, all the operations were performed at 4 ^{0}C.

2.2.2 Collection of Seeds

The ripe fruits of *Cassia fistula* commonly known as **Bandarlati** in our country were collected from Rajshahi University campus, Rajshahi, Bangladesh. The seeds were separated from the fruits, washed with water, dried under sunlight and used for experimental purposes.

2.2.3 Preparation of Fat Free Meals

The seeds were taken in a mortar and pounded uniformly into fine powder. This was then mixed uniformly with pre-cooled petroleum ether (b.p. 40 - 60 ^{0}C) in a homogenizer at 4 ^{0}C. The homogenate was filtered through a clean muslin cloth. The process was repeated at least for two times in order to obtain the homogenate quite fat-free. Finally the filtrate was further clarified by centrifugation at 8,000 g, 4 ^{0}C for 10 min. The precipitate thus obtained were collected and air dried at room temperature, and used for the extraction of protein.

2.2.4 Choice of Extracting Solvent

Proteins from fat free dry powder were extracted with five different extracting solvents under identical conditions. The suitable extracting solvent was selected from the ratio of absorbance at 280 nm and 260 nm as reported by Clark and Switzer (1977).

Table-2.1: Extraction of crude proteins from *Cassia fistula* seeds in different extracting solvents.

No. of observations	Extracting media	Amount of meal	Ratio of O. D. at 280 nm & 260 nm
1	1 % CH_3COOH	1 gm	0.9504
2	Tris-HCl buffer, pH - 8.4	1 gm	0.9701
3	Phosphate buffer, pH - 7.4	1 gm	0.9611
4	Distilled water	1 gm	0.9691
5	0.2 % NaCl in distilled water, pH 6.5	1 gm	0.9802

Distilled water containing 0.2 % NaCl, pH 6.5 was used as extracting solvent for preparation of crude protein extract from fat free dry powder as the highest ratio of absorbance at 280 nm and 260 nm was found (Table-2.1) in this solvent system.

2.2.5 Preparation of Crude Protein Extract

The fat free dry powder was mixed uniformly with pre-cooled distilled water containing 0.2 % NaCl, pH 6.5 (6 ml/gm of meal) and kept overnight at 4 ^{0}C with occasional gentle shaking. The suspension was then centrifuged at 8,000 g, 4 ^{0}C for 15 minutes. The clear supernatant was collected and adjusted to 100 % saturation by adding solid ammonium sulfate. The resulting precipitate was collected by centrifugation, dissolved in minimum volume of pre-cooled distilled water and dialyzed against distilled water for 24 hours with three changes and against 10 mM Tris-HCl buffer, pH 8.4 for 12 hours at 4 ^{0}C. After centrifugation the clear supernatant was used as crude protein extract.

2.2.6 Purification of Proteins

Gel Filtration

Gel filtration was carried out on Sephadex G-50 column. The crude protein extract was loaded onto the Sephadex G-50 column previously equilibrated with 10 mM Tris-HCl buffer, pH 8.4 and the proteins were eluted with the same buffer at 4 ^{0}C. The different fractions were collected and checked for hemagglutinating activity. Absorbance at 280 nm of each fraction as well as protein concentration by Folin-Lowry method (Lowry *et al.*, 1951) were also measured.

DEAE-Cellulose Chromatography

The active protein fraction obtained after gel filtration was dialyzed against distilled water for 12 hours and against 10 mM Tris-HCl buffer, pH 8.4 for over-night. It was then applied to DEAE-cellulose column previously equilibrated with 10 mM Tris-HCl buffer, pH 8.4. The absorbed proteins were eluted from the column by the linear gradient elution of NaCl (0.0-0.3 M) in the same buffer as well as stepwisely with the buffer containing different concentrations of NaCl. Analysis of each fraction was carried out similarly as described above.

Affinity Chromatography

For further purification of the protein, the active protein fractions obtained after DEAE-cellulose chromatography were dialyzed separately against distilled water for 12 hours and against 5 mM phosphate buffer saline, pH 7.2 for overnight and then were applied individually to Sepharose 4B column previously equilibrated with the same buffer at 4 ^{0}C. The adsorbed protein was eluted from the column by 5 mM phosphate buffer saline, pH 7.2 and then with the same buffer containing 0.2M galactose. Analysis of each fraction was also performed similarly as described above.

Polyacrylamide Disc Gel Electrophoresis

The homogeneity of the different fractions obtained after affinity chromatography was judged by polyacrylamide disc gel electrophoresis which was conducted at room temperature, pH 8.4 on 7.5% gels as described by Ornstein (1964). The gels were stained with 1% amidoblack in 7.5% acetic acid for an hour at room temperature and destaining was performed by washing the gels in 7% acetic acid (v/v) solution.

2.2.7 Characterization of Proteins

Molecular Weight Determination

Gel Filtration

The molecular weight of the purified proteins were determined by the gel filtration on Sephadex G-75 column (0.75 x 100 cm) using lysozyme, trypsin inhibitor, egg albumin and bovine serum albumin (BSA) as marker proteins according to the method of Andrews (1965).

SDS-PAGE

In the present study, the molecular weight of the purified proteins was also estimated by SDS-polyacrylamide gel electrophoresis on 10 % acrylamide gel as described by Weber and Osborn (1969) and the marker proteins used were same as those used in the gel filtration. The proteins were stained with Coomassie Brilliant blue R-250 and the destaining was performed by 7 % acetic acid solution.

Hemagglutination Studies

Hemagglutinating activity was assayed by serial dilution technique using albino rat red blood cells (RBC) as described by Lin *et al.* (1981). Protein solution (0.2 ml) in 5 mM phosphate buffer saline (PBS), pH 7.2 was mixed with 0.2 ml of 4%

(w/v) RBC and the mixture was incubated at 37 ^{0}C for 1 hour. A control containing 0.2 ml PBS, instead of protein solution and 0.2 ml cell suspension was used as reference. The degree of hemagglutination was observed under microscope and the results were recorded as 3^+, 2^+, 1^+, and ± .

The agglutinating activity was expressed as the titre, the reciprocal of the lowest concentration at which visible agglutination could be detected. The specific activity was expressed as the titre per mg protein.

Hemagglutination Inhibition Studies

The hemagglutination inhibition test was performed in the presence of different sugars. Protein solutions (0.1ml) containing minimum concentration of protein needed for visible agglutination were added to 0.1 ml sugar solutions of various concentrations and mixed gently. The mixture was then mixed with 0.2 ml of 4% RBC in PBS and incubated at 37 ^{0}C for 1 hour. Reactions were compared with a positive control (0.1 ml protein solution +0.1 ml PBS + 0.2 ml 4 % RBC) and a negative control (0.2 ml PBS + 0.2 ml 4 % RBC) as reported by Atkinson and Trust (1980).

Protein Concentration

The concentration of protein was measured by the method of Lowry *et al.* (1951) using BSA as the standard and the proteins in column elute fractions were also monitored spectrophotometrically at 280 nm.

Analysis of Carbohydrate

The presence of sugar in the purified proteins was detected by periodic acid Schiff's method (Anthony and Andrews, 1978) and the percentage of sugar was estimated by phenol-sulphuric acid method (Dubois *et al.*,1956) using D-glucose as the standard.

For identification of sugars, the protein solutions were hydrolyzed with 4M HCl for 4 hours at 100 ^{0}C under vacuum. The sugar components of the protein were then determined by the one dimensional thin layer chromatography (TLC) method as described by Touchstone and Dobbins (1978) using different standard sugars. The chromatogram was developed with a solvent system of isopropanol, acetic acid and water (3:1:1, v/v/v) in ascending manner and after drying, sprayed with aniline-phthalate solution. The chromato-spots were identified by comparing the R_f values with those of the standard sugars.

Brine Shrimp Lethality

Cytotoxicity was studied using Brine shrimp eggs. Shrimp eggs were placed in one side of a small tank divided by a net containing sea water (3.8% NaCl solution) for hatching. In the other side of the tank a light source was placed in order to attract the nauplii. Two days were allowed to hatch all the eggs and in this period the nauplii were also sufficiently matured for experiment as depicted by Mayer *et al.* (1982).

From the stock solutions of the protein samples, specific volumes were transferred to the different vials containing 10 living shrimps and then sea water was added to make the volume upto 5 ml in each vial. The final concentration of the sample in the vials became 2, 4, 8, 16, and 32 μg/ml respectively. Three experiments were carried out for the same concentration to get more accurate result and a control experiment was performed similarly taking 10 living shrimps in 5ml seawater.

After 24 hours incubation, the vials were observed and the number of deaths in each vial was counted using a magnifying glass. From this data, the mean percentage of mortality of the nauplii was calculated at each concentration.

Ultraviolet Absorption Spectra

The ultraviolet absorption spectra of the proteins were recorded in aqueous solution with a Shimadzu Model UV-180 Double Beam Spectrophotometer at room temperature.

2.3 Results and Discussion

Results

Purification of *Cassia fistula* Seed Proteins

The 100 % ammonium sulfate saturated crude protein extract after dialysis against 10 mM Tris-HCl buffer, pH 8.4, was applied to a Sephadex G-50 column at 4^0C which was previously equilibrated with the same buffer. As shown in Figure-2.1, the proteins were eluted from the column as one main peak, fraction F-2 and another two small peaks, fractions F-1 and F-3. The active fraction, F-2 as indicated by solid bar was pooled, precipitated by ammonium sulfate and purified further by ion exchange chromatography on DEAE-cellulose. The fractions F-1 and F-3 were not used for further study as they contained small amount of protein as well as possessed no significant activity.

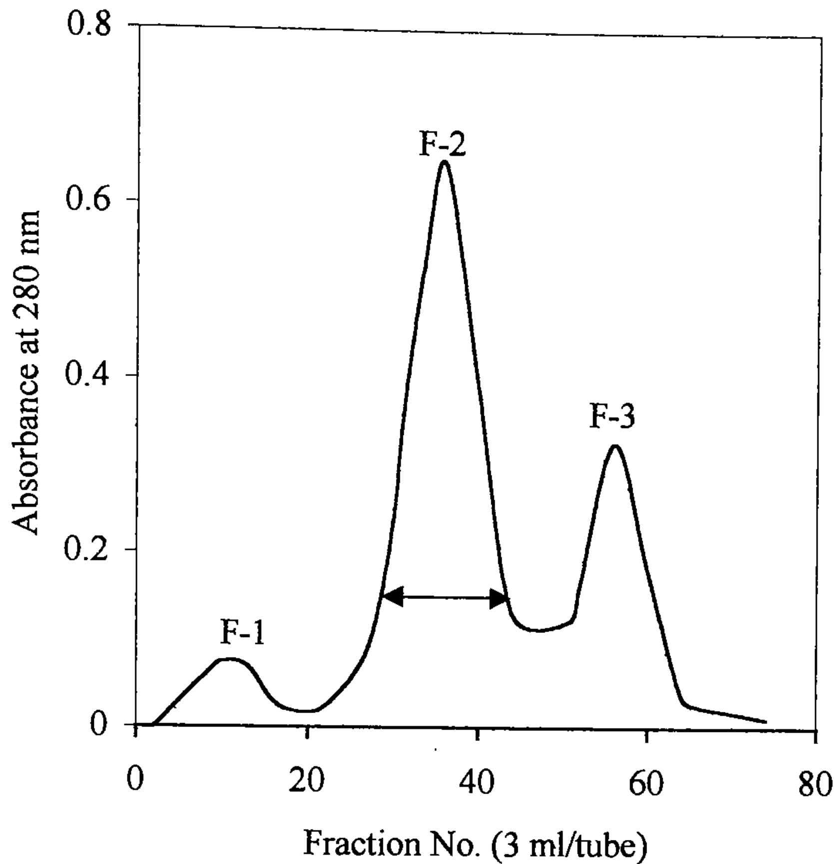


Figure-2.1: Gel filtration of 100 % $(NH_4)_2SO_4$ saturated crude protein extract on Sephadex G-50 column. The crude protein extract (30 mg) was applied to the column (2.5 x 100 cm), pre-equilibrated with 10 mM Tris-HCl buffer, pH 8.4 at 4^0C and developed with the same buffer. Flow rate: 24 ml / hour.

The ammonium sulfate precipitate of fraction F-2, obtained after centrifugation, was dissolved in minimum volume of distilled water and dialyzed against distilled water for 12 hours and against 10 mM Tris-HCl buffer, pH 8.4 at 4^0C for overnight with three changes of buffer. After removal of the insoluble material by centrifugation, the clear supernatant was applied to a DEAE-cellulose column at 4^0C, which was previously equilibrated with 10 mM Tris-HCl buffer, pH 8.4, and eluted by a linear gradient of NaCl from 0.0 to 0.3 M in the same buffer.

Figure-2.2 shows that the column bound proteins were eluted as a single but broad peak, indicating the presence of more than one component. In order to separate these components, the elution was carried out stepwisely with increasing concentrations of NaCl in the same buffer.

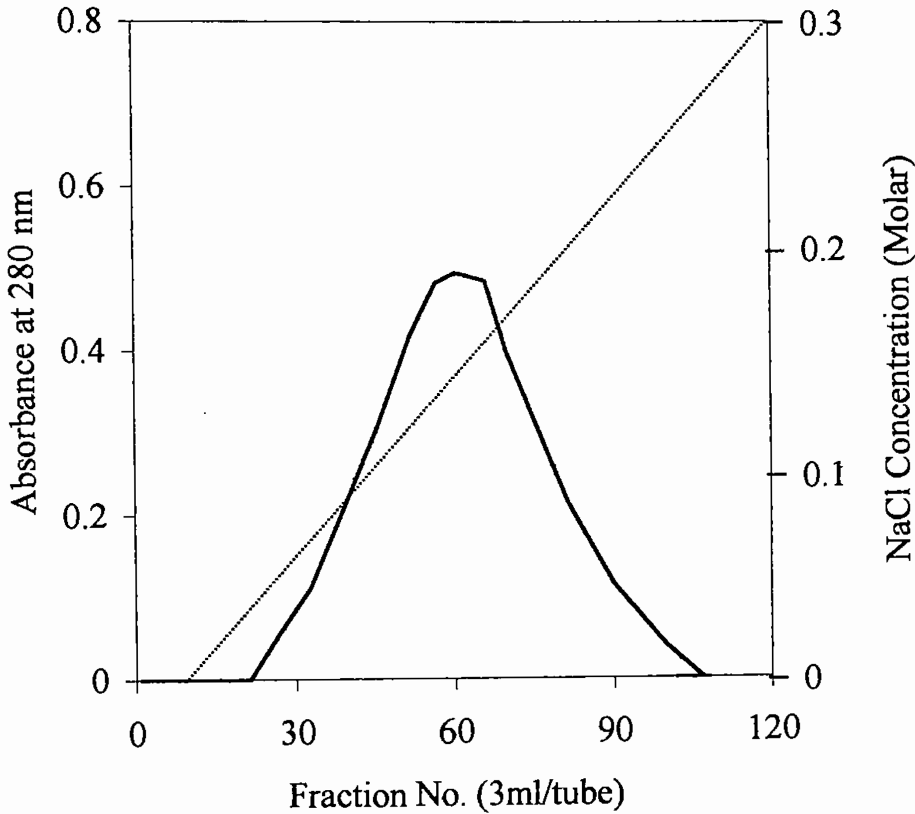


Figure-2.2: Ion exchange chromatography of fraction, F-2 on DEAE-cellulose. Fraction F-2 (21 mg), obtained from gel filtration, was applied to the column (2.1 x 24 cm), which was pre-equilibrated with 10 mM Tris-HCl buffer, pH 8.4 at 4^0C and eluted by a linear gradient of NaCl (0.0 to 0.3) in the same buffer. Flow rate: 45 ml/hour.

As shown in Figure-2.3 the components of F-2 fraction were separated into four different fractions, F-2a, F-2b, F-2c and F-2d, which were eluted with the same buffer containing 0.05, 0.10, 0.25 and 0.50 molar NaCl respectively. The areas as

indicated by solid bar for the active fractions F-2b, F-2c and F-2d were pooled separately and further purified by affinity chromatography on Sepharose-4B. On the other hand, fraction F-2a was discarded as it contained no significant hemagglutination activity.

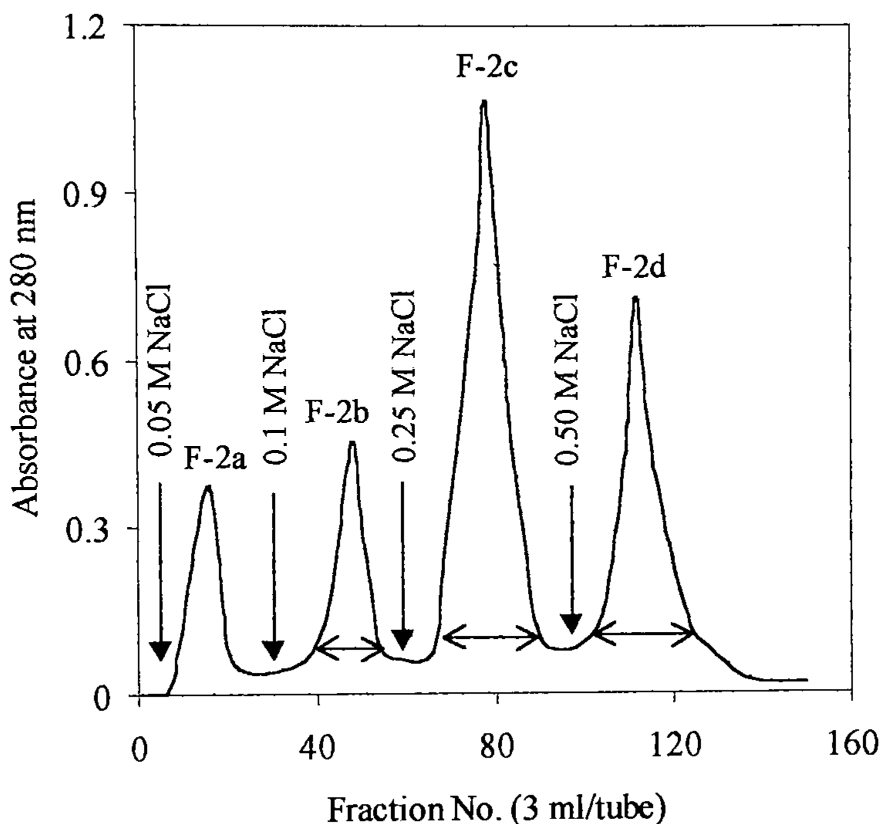


Figure-2.3: Ion exchange chromatography of fraction F-2 on DEAE-cellulose. Fraction F-2 (45 mg) obtained by gel filtration, was applied to the column (2.1 x 24 cm) pre-equilibrated with 10 mM Tris-HCl buffer, pH 8.4 at 4^0C and eluted by stepwise increases of NaCl concentration in the same buffer. Flow rate: 45 ml/hour.

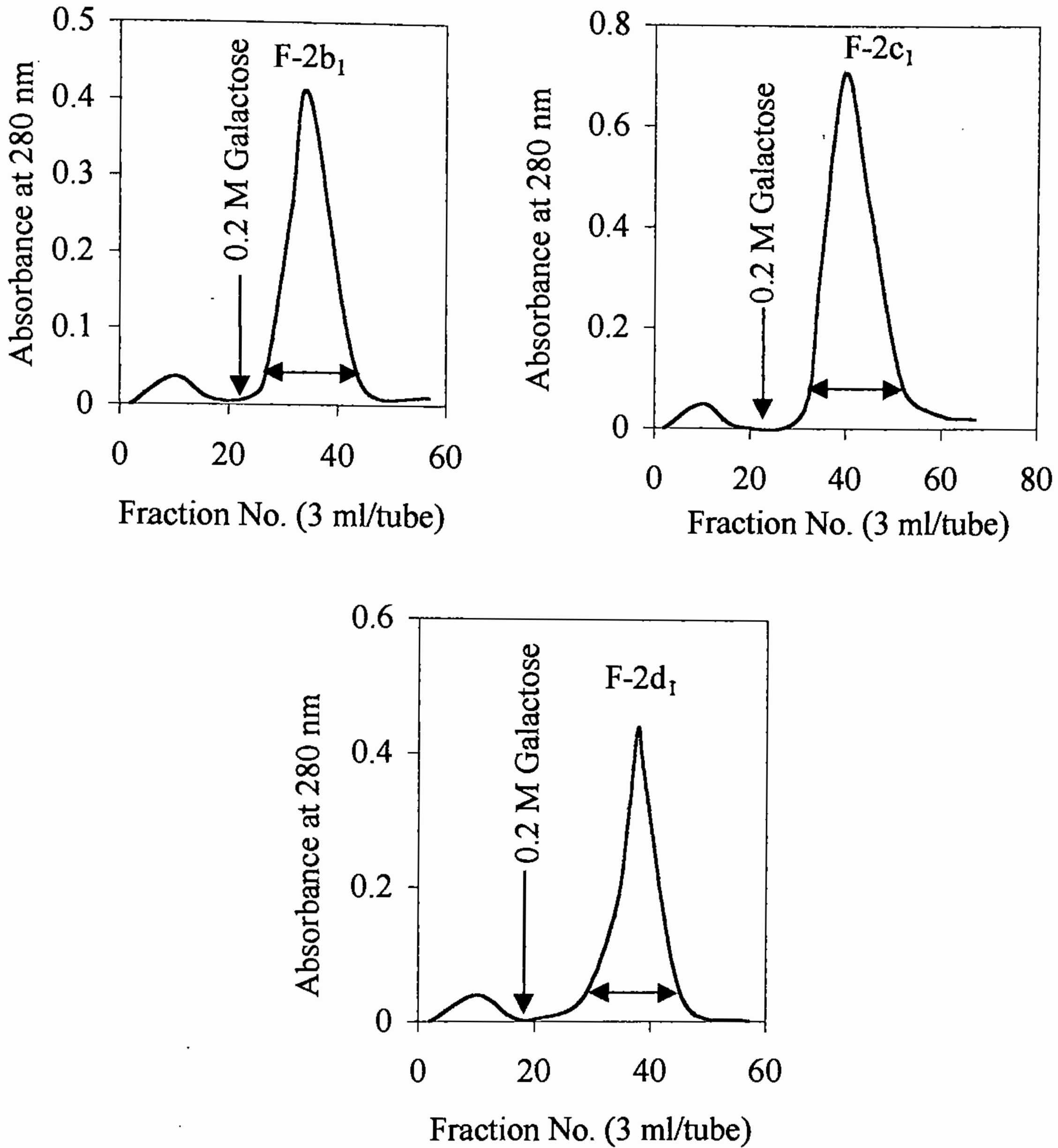


Figure-2.4: Affinity chromatography of fractions F-2b (6.08 mg), F-2c (10.38 mg) and F-2d (6.29 mg) obtained from DEAE-cellulose column, on Sepharose 4B. These fractions were applied individually to the column (1.25 x 10 cm) pre-equilibrated with 5 mM phosphate buffer saline, pH 7.2 at 4^0C and the absorbed proteins were eluted by the same buffer containing 0.2 molar galactose. Flow rate: 30ml/hr.

As shown in Figure-2.4, most of the proteins in the fractions were bound tightly on the affinity matrix and eluted mainly as one peak by the buffer containing 0.2 M galactose. The purities of the galactose specific fractions F-2b$_1$, F-2c$_1$ and F-2d$_1$ were checked by polyacrylamide disc gel electrophoresis and the photographic representations of the electrophoretic patterns are shown in Figure-2.5

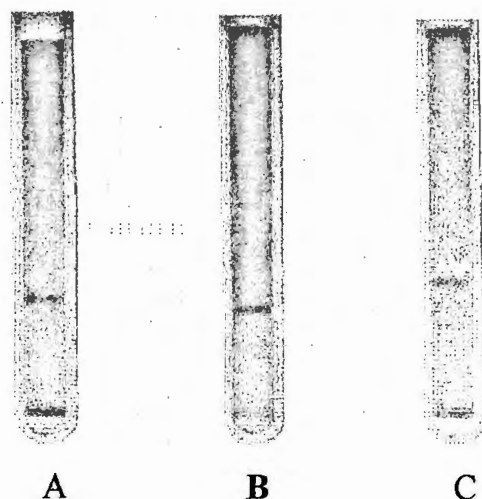


Figure-2.5: Polyacrylamide disc gel electrophoretic patterns of the purified proteins at room temperature on 7.5 % gel. A = F-2b$_1$, B = F-2c$_1$ and C = F-2d$_1$

It can be concluded from the findings that the fractions F-2b$_1$, F-2c$_1$ and F-2d$_1$ might be contained pure protein as they gave single band on the gel. All the three purified fractions also displayed hemagglutinating activity. A flow diagram for overall purification steps of proteins from *Cassia fistula* seed is given in Figure-2.6.

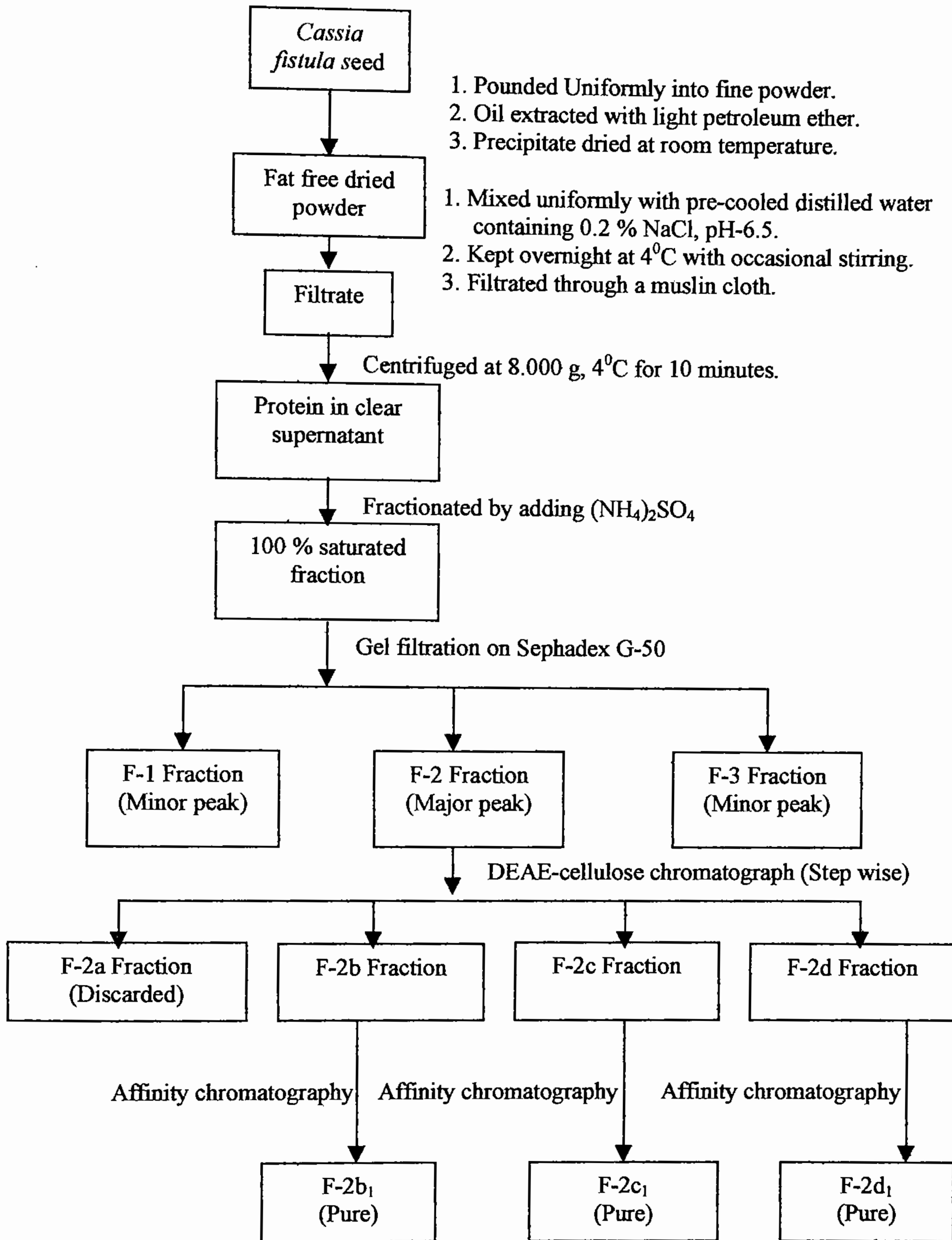


Figure-2.6: Flow diagram for purification of proteins from *cassia fistula* seeds.

The overall purification data of *Cassia fistula* seed proteins were summarized in Table-2.2. From the Table, it was evident that the specific activities of the different protein fractions were increased at each purification step and the fraction F-2c_1 showed maximum hemagglutinating activity with a purification fold of 11.59, while the fraction, F-2b_1 and F-2d_1 showed the purification fold of 8.93 and 9.69 respectively. Although the yield of these proteins were found to be decreased after different purification steps and more than 96 % of extracted protein was destroyed during the purification processes but the purification fold of the proteins were increased after each subsequent purification step. It may suggest from the result that the decrease in yield might be due to the denaturation of proteins during the lengthy purification procedures or some other reasons.

Table-2.2: Purification of *cassia fistula* seed proteins

Fractions		Total protein (mg)	Hemagglutination activity (titre)	Specific activity (titre/mg)	Yield (%)	Purification fold
Crude extract		580	2494	4.30	100	1.00
100 % $(NH_4)_2SO_4$ saturation		222	1860	8.37	74.57	1.94
After gel filtration		90	1358	15.08	54.45	3.50
DEAE-Cellulose fractions	F-2b	6.08	209	34.37	8.38	7.99
	F-2c	10.38	468	45.08	18.76	10.48
	F-2d	6.29	240	38.15	9.62	8.87
Affinity Chromatography	F-2b$_1$	4.92	189	38.41	7.58	8.93
	F-2c$_1$	8.87	442	49.83	17.72	11.59
	F-2d$_1$	5.28	220	41.66	8.82	9.69

Hemagglutination (Hg) activity (titre) = Reciprocal of lowest concentration showing visible hemagglutination.

$$\text{Yield} = \frac{\text{Observed Hg-activity}}{\text{Initial Hg-activity}} \times 100$$

$$\text{Fold} = \frac{\text{Observed specific activity}}{\text{Initial specific activity}}$$

Molecular Weight of the Proteins

Gel Filtration

The molecular weights of purified *Cassia fistula* seed proteins were determined by gel filtration on Sephadex G-75 column using lysozyme (MW=14,600), trypsin inhibitor (MW=20,000), egg albumin(MW=45,000) and bovine serum albumin (MW=67,000) as marker proteins under identical experimental conditions. The molecular weight of the unknown proteins was calculated from the standard curve, which was constructed by plotting the molecular weight against elution volume of the marker proteins and were found to be about 37,000, 42,400 and 46,000 for F-$2b_1$ (*Cassia fistula* seed lectin-1, i.e., CSL-1), F-$2c_1$ (*Cassia fistula* seed lectin-2, i.e., CSL-2) and F-$2d_1$ (*Cassia fistula* seed lectin-3, i.e., CSL-3) respectively (Figure-2.7).

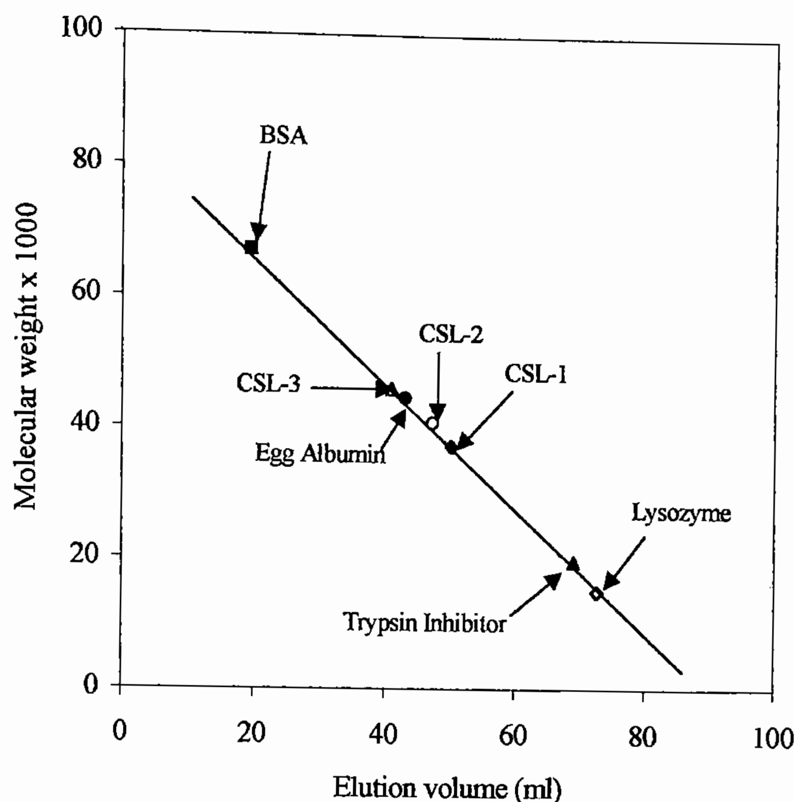


Figure-2.7: Standard curve for determination of molecular weight by gel filtration on Sephadex G-75. Proteins were applied to a Sephadex G-75 column (1.6 x 90 cm) pre-equilibrated with 10 mM Tris-HCl buffer, pH 8.4 at 4^0C and eluted with the same buffer. Flow rate: 15 ml/hr.

SDS-PAGE

The molecular weight of the purified proteins was also determined by SDS-polyacrylamide gel electrophoresis at pH 7.2 on 10 % acrylamide gel using lysozyme, trypsin inhibitor, egg albumin and bovine serum albumin as reference proteins. The molecular weight of the proteins was calculated from the standard curve of reference proteins, which was constructed by plotting the molecular weight against relative mobility of the reference proteins on gel after electrophoresis. Molecular weights were determined to be about 37500, 42000 and 46500 for CSL-1, CSL-2 and CSL-3 respectively (Figure-2.8).

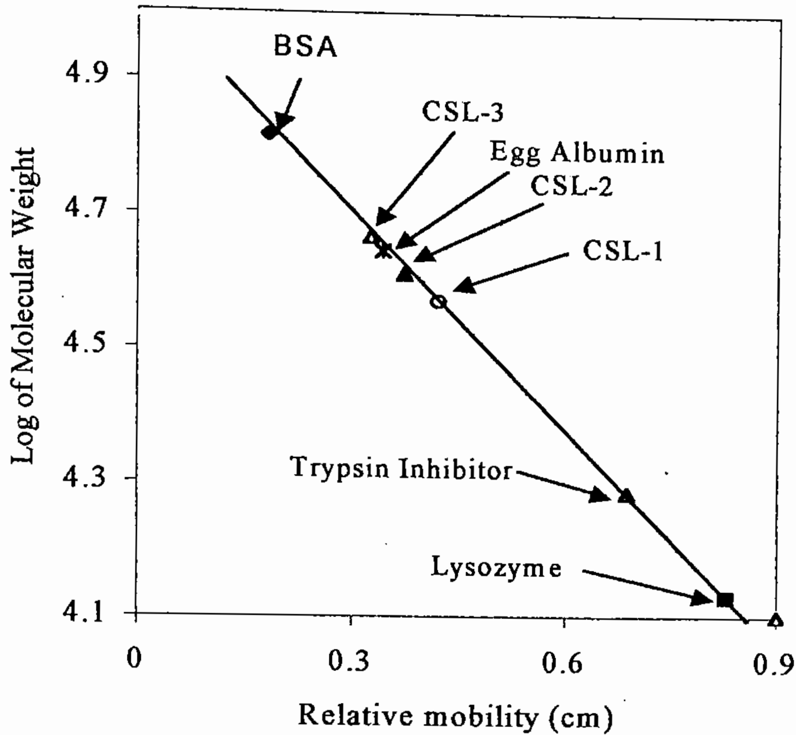


Figure-2.8: Standard curve for determination of molecular weight by SDS-polyacrylamide gel electrophoresis.

Hemagglutinating Activities

All the lectins agglutinated specifically the albino rat red blood cells (RBC). Table-2.3 shows the degree of hemagglutination of albino rat red blood cells by CSL-1, CSL-2 and CSL-3 and the photographic representations of hemagglutination are shown in Figure-2.9, 2.10 and 2.11 respectively. The absorbance at 280 nm needed for visible agglutination was considered as minimum hemagglutination dose (MHD) and were found to be 0.0361, 0.0242 and 0.0328 for CSL-1, CSL-2 and CSL-3 respectively.

Table-2.3 Hemagglutinating activities of *Cassia fistula* seed proteins with 4% red blood cells (RBC) from albino rat.

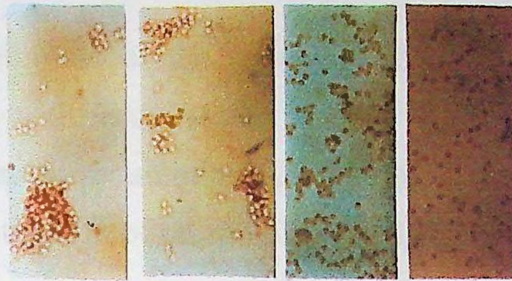
Protein samples	Absorbance at 280 nm	Degree of Hemagglutination
CSL-1	0.1200	3^+
	0.0900	2^+
	0.0650	1^+
	0.0361	±
CSL-2	0.0804	3^+
	0.0620	2^+
	0.0391	1^+
	0.0242	±
CSL-3	0.1012	3^+
	0.0820	2^+
	0.0591	1^+
	0.0328	±

3^+ Indicates complete aggregation of almost all the cells.

2^+ Indicates lesser degree of agglutination where smaller number of cells remained free.

1^+ Indicates all the cells were present in small aggregation of varying sizes.

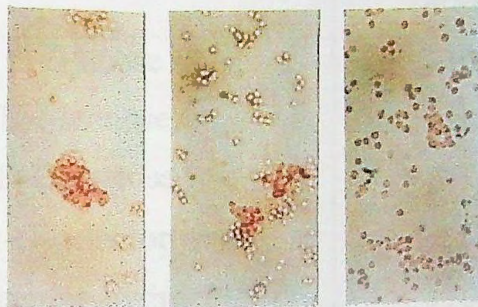
± Indicates major cells were present in aggregates.



A B C D

Figure-2.9: Agglutination of albino rat red blood cells by CSL-1

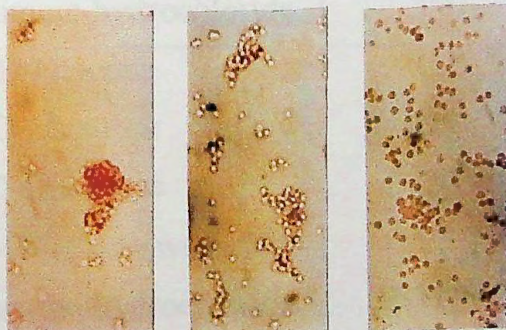
$A = 3^+$, $B = 2^+$, $C = 1^+$ and D = Control



A B C

Figure-2.10: Agglutination of albino rat red blood cells by CSL-2

$A = 3^+$ $B = 2^+$ and $C = 1^+$



A B C

Figure-2.11: Agglutination of albino rat red blood cells by CSL-3

$A = 3^+$ $B = 2^+$ and $C = 1^+$

Hemagglutination Inhibition Studies

Hemagglutination Inhibitions of the proteins, CSL-1, CSL-2 and CSL-3 were performed in presence of different sugars and the results were depicted in Table-2.4. Galactose and galactose containing saccharide are found to be highly specific for inhibiting the agglutination of rat red blood cells by all the lectins.

Table-2.4: Hemagglutination Inhibition assay of *Cassia fistula* seed lectins by different sugars.

Proteins	Sugars	Concentration (mM)	Hemagglutination
CSL-1	D-Glucose	110	NI
	D-Mannose	110	NI
	D-Galactose	20	I
	Methyl -α-D-galactopyranoside	25	I
	D-Glucosamine-hydrochloride	110	NI
CSL-2	D-Glucose	80	I
	D-Mannose	110	NI
	D-Galactose	20	I
	Methyl -α-D-galactopyranoside	30	I
	D-Glucosamine-hydrochloride	100	NI
CSL-3	D-Glucose	110	NI
	D-Mannose	85	I
	D-Galactose	20	I
	Methyl -α-D-galactopyranoside	25	I
	D-Glucosamine-hydrochloride	110	NI

I-Inhibition NI-No inhibition

Protein Concentration

The absorbance of 1.0 at 280 nm for CSL-1, CSL-2 and CSL-3 were found to be equal to 0.72, 0.83 and 0.73 mg of proteins, respectively as determined the concentration of protein by the Lowry method (Table-2.5).

Table-2.5: Optical density (O. D.) and concentration relationship of the proteins.

Proteins	O. D. of proteins at 280 nm	Amount of proteins (mg)
CSL-1	1.0	0.72
CSL-2	1.0	0.83
CSL-3	1.0	0.73

Analysis of Carbohydrate

The presence of sugars in the proteins was confirmed by periodic acid schiff (PAS) staining method. It was found that all the proteins produced pinkish red band on polyacrylamide gel, when gels were stained with PAS reagent after electrophoresis. The neutral sugar contents as estimated by phenol-sulphuric acid method, of the lectins, CSL-1, CSL-2 and CSL-3 were estimated to be 3.5, 3.1 and 2.0 % respectively. The sugar composition of the lectins as identified by one dimensional thin layer chromatography (TLC) was found to be galactose for CSL-1, galactose and glucose for CSL-2, and galactose and mannose for CSL-3.

Cytotoxic effects

All the three lectins exhibited significant toxic effect on brine shrimp lethality bioassay. The mortality rate of brine shrimp nauplii was found to increase with the increase in concentration of the lectins and a plot of log of concentration vs. percent of mortality gave almost linear correlation (Figure-2.12).

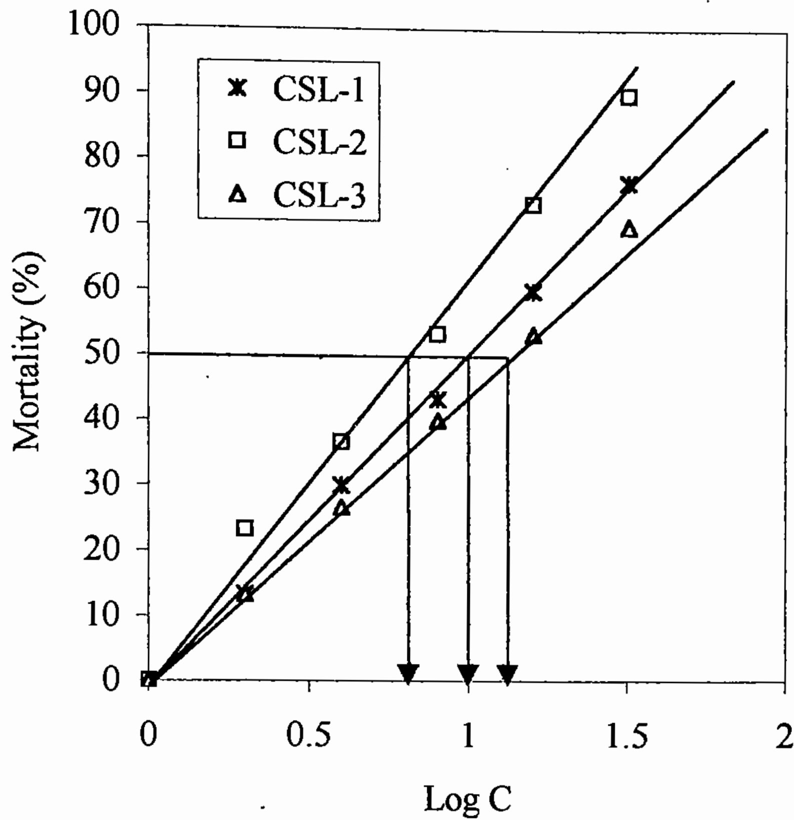


Figure-2.12: Determination of LC_{50} of *Cassia fistula* seed lectins

From the graph, the LC_{50} (concentration at which 50 % mortality of the nauplii occurs) as estimated by the extrapolation was found to be 10.47 μg/ml for CSL-1, 6.68 μg/ml for CSL-2 and 13.33 μg/ml for CSL-3 (Table-2.6).

Table-2.6: Effect of *Cassia fistula* seed lectins on brine shrimp lethality bioassay

Test samples	Conc. (μg/ml)	Log conc. (Log C)	No. of shrimp taken	No. of shrimp death			Avarage. No. of death	Mortality (%)	LC_{50} (μg/ ml)
				Vial 1	Vial 2	Vial 3			
Nil (Control)	0	0	10	0	0	0	0	0	
CSL-1	2	0.3010	10	1	2	1	1.333	13.33	10.47
	4	0.6020	10	3	3	3	3.000	30.00	
	8	0.9030	10	4	4	5	4.333	43.33	
	16	1.2041	10	6	6	6	6.000	60.00	
	32	1.5051	10	8	8	7	7.666	76.66	
CSL-2	2	0.3010	10	2	2	2	2.000	20.00	6.68
	4	0.6020	10	3	4	4	3.666	36.66	
	8	0.9030	10	5	5	6	5.333	53.33	
	16	1.2041	10	7	7	8	7.333	73.33	
	32	1.5051	10	9	9	9	9.000	90.00	
CSL-3	2	0.3010	10	2	1	1	1.333	13.33	13.33
	4	0.6020	10	3	2	3	2.666	26.66	
	8	0.9030	10	4	4	4	4.000	40.00	
	16	1.2041	10	5	5	6	5.333	53.33	
	32	1.5051	10	7	7	7	7.000	70.00	

Ultraviolet Absorption Spectra

The ultraviolet absorption spectra of the proteins were recorded in aqueous solution with a Shimadzu Model UV-180 Double Beam Spectrophotometer at room temperature. The purified proteins CSL-1, CSL-2 and CSL-3 in aqueous

solution gave absorption maxima around 274, 278 and 276 nm, and minima around 243, 248 and 246 nm respectively (Figure-2.13).

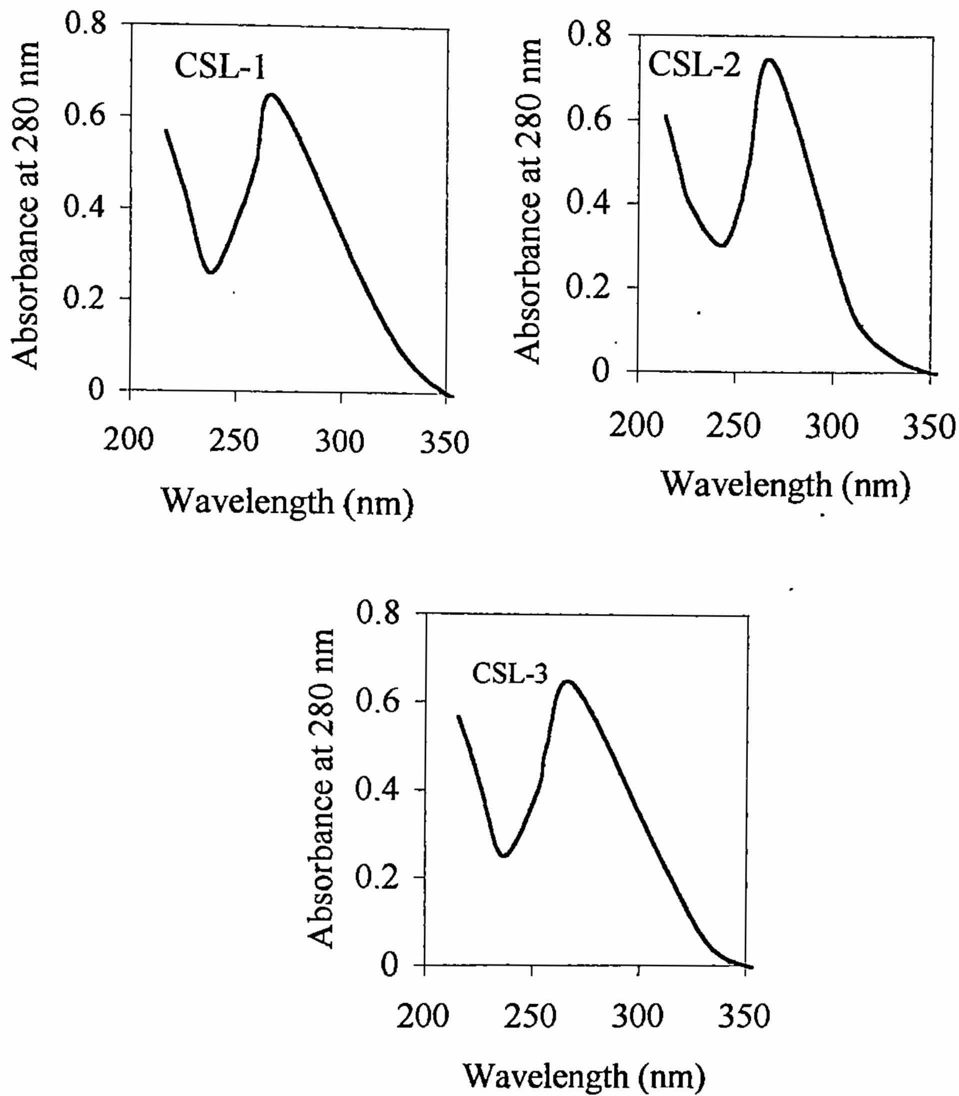


Figure-2.13: Ultraviolet absorption spectra of CSL-1, CSL-2 and CSL-3 lectins in aqueous solution.

Discussion

Three proteins have been isolated and purified from the crude protein extract of the *Cassia fistula* seeds with a purification fold of about 30.21 fold as compared to

that of the crude protein extract. All these proteins are glycoproteins in nature as they gave orange yellow color in the presence of phenol-sulfuric acid. The presence of sugar in the lectins was further confirmed by the pinkish-red band produced on polyacrylamide gel stained with periodic acid Schiff's staining reagent after electrophoresis.

The agglutinations of rat red blood cells by the lectins CSL-1, CSL-2 and CSL-3 were inhibited specifically in the presence of galactose and galactose containing saccharide. This finding was further supported by the facts that all the three lectins showed binding affinity to Sepharose 4B during purification processes. It is concluded from the above findings that *Cassia fistula* seed contained at least three lectins that are specific for D-galactose.

Although the purified lectins from *Cassia fistula* seeds are similar in their sugar specificities, but quite differ from each other in their molecular weight, neutral sugar content and sugar composition. All the three lectins moved as a single band with almost similar mobilities on polyacrylamide gel and the molecular weights of the CSL-1, CSL-2 and CSL-3 were found to be about 37250, 42200 and 46250 respectively by gel filtration and SDS-PAGE. Further study is going on to determine the subunit structure of the lectins.

Like seed lectins from *Abrus precatorius, Ricinus communis, T. anguina and Momordica charantia,* all three lectins, purified in this study are cytotoxic in nature as they affect significantly the mortality rate of brine shrimp.

In conclusion, the purified lectins, CSL-1, CSL-2 and CSL-3, besides being specific for rat red blood cells agglutination, can be added as an addition of members to the list of the purified member of galactose binding lectins. Among the lectins purified, CSL-2 is found to be highly toxic followed by CSL-1 and then CSL-3.

Chapter-3

Effect of Physico-chemical Agents on the Hemagglutinating Activities of *Cassia fistula* Seed Lectins

Effect of Physico-chemical Agents on the Hemagglutinating Activities of *Cassia fistula* Seed Lectins.

3.1 Introduction

The conformation of a native or highly organized protein reflects a delicate balance among a variety of interaction forces both within the folded protein interiors and with surrounding solvent. If the protein's solvent environment is perturbed, the protein's native conformation can be disrupted, with a resulting loss of function and the production of a partially unfolded or denatured protein. The conditions giving rise to partial denaturation and consequent loss of function may be subtle such as small change in pH, temperature and ionic strength on dielectric constant of the medium. Conversely, prolonged boiling or exposure to thiol-containing compounds and denaturants such as sodium dodecyl sulfate or to hydrogen bond-breaking reagents such as urea or guanidine hydrochloride may be required for complete denaturation (Zubey, 1988).

The three dimensional structure of a protein is governed by its primary structure and its environment. The organized native structure (conformation) of a protein is known to be affected from the effect of external environmental changes such as temperature, acidity, urea or denaturant solutions and a number of other chemicals. In structural studies of proteins, it is often necessary to established conditions for reversible denaturation. The choice of denaturation condition depends on the stability of the protein of interest. Among the techniques used for reversible denaturation are lowering of the pH (Itano & Singer,1958), freezing and thawing in concentration of salt (Market, 1963) and adding denaturants such as urea and

guanidine-HCl (Chilson *et al.*,1964 & 1965, Meighen and Schachman, 1970 a & 1970 b).

Cassia fistula seed lectins are glycoproteins in nature. All the three proteins, purified from its seeds specifically agglutinate rat red blood cells and may also be used to perform many other biological activities. In the present study, the lectins have been subjected to various physical and chemical treatments, and their effects on the hemagglutination activities were analyzed. The study is expected to provide important information regarding some of the physico-chemical properties such as pH stability, thermal stability and the stability of the lectins towards denaturing agents. The experimental results also give indications in establishing conditions for chemical modification which in turn, are expected to be helpful in understanding the relationship between structure and function.

3.2 Materials and Methods.

3.2.1 Chemicals

Acetic acid and urea were the products of British Drug House (BDH), Poole, England. Guanidine-HCL was the product of Bio-Rad Laboratories, Richmond, California, USA. All other reagents used were of analytical grade.

3.2.2 Hemagglutinating Activity

After various physico-chemical treatments of protein solutions as depicted below, the Hemagglutinating activity was determined according to the method of Lin *et al.*, (1981) using 4% albino rat red blood cells, as described in Chapter 2.

3.2.3 Physical Treatments of the Lectins

Effect of pH

Lectin solutions in 50 mM respective buffers possessing pH ranges from 3.0-9.5 were incubated for 10 hours at 28 ^{0}C. The hemagglutinating activity retained was determined after dialysis the lectin solutions against 5 mM phosphate buffer saline, pH 7.2 for 18 hours at 4^0 C

Effect of Temperature

Lectin solutions in 5 mM phosphate buffer saline, pH 7.2 were heated at various temperatures for 1 hour using a temperature controlled waterbath. After cooling the heated lectin solutions in an ice bath, the hemagglutinating activity was determined.

3.2.4 Chemical Treatments of the Lectins

Treatment with Acetic Acid

Lectin solutions (200 µl) in 5 mM phosphate buffer saline, pH 7.2, were mixed with acetic acid at different concentrations. After an incubation period of 1 hour at 4°C, the lectin solutions were dialyzed against 5 mM phosphate buffer saline, pH 7.2 for 12 hours at 4°C, and then hemagglutinating activity was determined.

Treatment with Urea

Solid urea of different concentrations was added to the lectins in 10 mM Tris-HCl buffer, pH 8.2. The solutions were incubated at 15 ^{0}C for 12 hours and then dialyzed against 5 mM phosphate buffer saline, pH 7.2 for overnight at 4^{0}C to remove urea. The hemagglutinating activity of the dialyzed solution was determined.

Treatment with Guanidine-HCl

To the lectin solutions in 10 mM Tris-HCl buffer, pH 8.2, was added solid guanidine-HCl of different concentrations. After incubation at 20 ^{0}C for 12 hours, the solutions were dialyzed against 5 mM phosphate buffer saline, pH 7.2 for 12 hours at 4 ^{0}C and the hemagglutinating activity was estimated.

3.3 Results and Discussion

Results

Effect of pH on Hemagglutinating Activity

The effect of pH on the hemagglutinating activities of CSL-1, CSL-2 and CSL-3 are depicted in Table-3.1. Results indicated that the biological activities of the three lectins were remarkably influenced by the pH changes. The hemagglutinating activities of the lectins were found to be much higher in mild basic pH region than the acidic pH values and the maximum activities were observed between pH 7.2 to 7.5. Further, the hemagglutinating activities decreased rapidly at or above pH 9.5 as well as at or below pH 6.0.

Table-3.1 : Hemagglutinating activities of *Cassia fistula* seed lectins at different pH values.

Buffer composition	pH	Relative hemagglutinating activity (%)		
		CSL-1	CSL-2	CSL-3
AcONa - HCl	3	5	10	10
AcONa – CH_3COOH	4.0	15	20	20
AcONa – CH_3COOH	5.0	25	30	40
NaH_2PO_4 – Na_2HPO_4	6.0	45	50	60
NaH_2PO_4 – Na_2HPO_4	6.5	70	75	80
NaH_2PO_4 – Na_2HPO_4	7.2	100	100	100
NaH_2PO_4 – Na_2HPO_4	7.5	90	100	100
NaH_2PO_4 – Na_2HPO_4	8.0	70	80	85
$Na_2B_4O_7$ – HCl	9.5	20	30	25

Effect of Temperature on Hemagglutinating Activity

As mentioned in Table-3.2, the hemagglutinating activities of CSL-1, CSL-2 and CSL-3 were affected remarkably by temperature changes and were found to be maximum around the temperature 20-35 ^{0}C. Again, the hemagglutinating activities of the lectins decreased rapidly with further rise of temperature and the activities were destroyed completely around 70 ^{0}C.

Table-3.2 : Effect of temperature on Hemagglutinating activities of *Cassia fistula* seed lectins.

Temperature (^{0}C)	Relative hemagglutinating activity (%)		
	CSL-1	CSL-2	CSL-3
20	100	100	100
25	100	100	100
30	100	100	100
35	95	100	100
40	85	90	85
50	55	65	65
60	20	35	30
70	0	0	0

Effect of Acetic Acid on Hemagglutinating Activity

As shown in Table-3.3, the hemagglutinating activities of CSL-1, CSL-2 and CSL-3 were fully retained even after treatment with 0.5 % acetic acid and were then decreased sequentially with further increase in acetic acid concentration. Further the results clearly demonstrated that all the three lectins destroyed about 50 % activities in the presence of 10 % acetic acid but lost their activities completely after treatment with 30 % acetic acid.

Table-3.3 : Effect of acetic acid at different concentrations on Hemagglutinating activities of *Cassia fistula* seed lectins.

Concentration of acetic acid (%)	Relative hemagglutinating activity (%)		
	CSL-1	CSL-2	CSL-3
0.0	100	100	100
0.5	100	100	100
2.5	80	90	85
5	70	80	70
10	40	50	50
20	15	25	20
30	00	00	00

Effect of Urea on Hemagglutinating Activity

The hemagglutinating activities of CSL-1, CSL-2 and CSL-3 were decreased sequentially with increased in urea concentration and the activities were abolished almost completely after treatment with 9M urea (Table-3.4). The results also indicated that CSL-3 is slightly more sensitive to urea than those of CSL-1 and CSL-2.

Table-3.4: Effect of urea at different concentrations on Hemagglutinating activities of *Cassia fistula* seed lectins.

Concentration of urea (Mole)	Relative hemagglutinating activity (%)		
	CSL-1	CSL-2	CSL-3
0	100	100	100
1	100	100	100
2	80	90	75
4	65	70	60
6	40	40	35
8	20	20	10
9	00	00	00

Effect of Guanidine-HCl on Hemagglutinating Activity

The hemagglutinating activities of CSL-1, CSL-2 and CSL-3 were markedly affected after treatment with guanidine-hydrochloride. The results as depicted in Table-3.5 indicated that CSL-1, CSL-2 and CSL-3 retained only 10 %, 20 % and 15 % activities respectively after treatment with 2 M guanidine-hydrochloride. The activities of the lectins were abolished completely after treatment with 6M guanidine-HCl.

Table-3.5: Effect of Guanidine-HCl at different concentrations on hemagglutinating activities of *Cassia fistula* seed lectins.

Concentration of Guanidine-HCl (Mole)	Relative hemagglutinating activity (%)		
	CSL-1	CSL-2	CSL-3
0.0	100	100	100
0.25	85	90	85
0.50	60	70	55
1.0	25	35	25
2.0	10	20	15
4.0	5	10	10
6.0	00	00	00

Discussion

The present study has been carried out to determine the stability of CSL-1, CSL-2 and CSL-3 by using physical and chemical means. The present data concluded that the hemagglutinating activities of *Cassia fistula* seed lectins were affected with the changes of pH as well as temperature. Results showed that CSL-1, CSL-2 and CSL-3 were more stable in slightly basic pH (i.e. pH 7.2-7.5) than the acidic pH region. Activities of all the three lectins were found to be active upto 35 ^{0}C, and the activities then decreased rapidly with further rise of temperature suggesting denaturation or disorganization of the structure of lectins at higher temperature.

The biological activities of CSL-1, CSL-2 and CSL-3 lectins were also followed after treatment with acetic acid. Results showed good correlation with those obtained by previous experiments i. e. changes of pH and rise of temperature. All the three lectins were found to be inactive almost completely after treatment with

30 % acetic acid, which might be due to denaturation or destruction of the native structure of the lectins. The hemagglutinating activities of *Cassia fistula* seed lectins were affected sequentially with the increase in concentration of denaturant such as urea and guanidine-HCl. It was found that the lectins CSL-1, CSL-2 and CSL-3 were more sensitive to guanidine-HCl than urea. The three lectins were inactivated almost completely after treatment with 6M guanidine-HCl whereas in presence of 6M urea, CSL-1, CSL-2 and CSL-3 showed 40 %, 40 % and 35 % activities respectively.

Chapter-4

Physico-chemical Investigations of *Cassia fistula* Seed Oil

Physico-chemical Investigations of *Cassia fistula* Seed Oil

4.1 Introduction

Fats and oils belong to the group of naturally occurring compounds called Lipids (Greek, lipos=fat.). Lipids are those constituents of animals and plants, which are soluble in organic solvents such as ether, chloroform, benzene, hexane etc., but insoluble in water. Fats and oils are the most important lipids found in nature, which yield long-chain fatty acid and glycerol upon hydrolysis.

Fats and oils are one of the three major " Food factors" needed for human body, the other two being protein and carbohydrate. They are widely distributed in foods and are of great nutritional values. They provide concentrated reserve of energy in animal body for maintaining optimum body temperature. One gram of metabolized fat or oil yields 9 k-cal., while the corresponding values of carbohydrate and protein are 4 k-cal and 5.5 k-cal respectively (Bahl and Arun, 1994). At present the human race uses an estimated 40 million tones of fats and oils in a year which reflects both their nutritional and industrial importance; and it is increasing day by day in a very large magnitude.

Seed oils from plants such as the olive, palm, coconut and peanut are exploited commercially and are used as edible fats, for soap manufacture and paint industry. Plant fats unlike animal fats, are rich in unsaturated fatty acids and there is evidence that some of these may be essential as a dietary requirement in human. The fatty acids of jute (*C. capsularis* and *C. olitorius)* seed oil have been analyzed (Montgomery *et. al.,* 1983). The oil contained varying amounts of palmitic,

stearic, arachidic, oleic, linoleic and C_{20} acids. Besides, the fatty acid composition of lipids from the tissue cultures as well as from the seeds of *C. capsularis* and *C. olitorius* varieties of jute plant (Stoller *et. al.*, 1974) were determined. The determination of fatty acid constituents in many other plant materials such as flue-cured tobacco (Ellington *et. al.*, 1987), millet seeds (Pansu *et. al.*, 1981), sunflower oil (Martel, 1981), mustard oil (Durante Franca Badoloto, *et. al.*, 1983), was reported.

The above mentioned investigations and reports make me much interests to carry out research work on fats and oils. There are a lot of plant materials like *Cassia fistula* seed oil yet to be investigated.

4.2 Materials and Methods

4.2.1 Materials

Solvents used during this work were purified by distillation at the boiling point of the respective solvents. A GCD Pye Unicam gas chromatograph equipped with a flame ionization detector was used to determine fatty acid composition of oil. All other chemicals used were of analytical grade.

4.2.2 Collection and Processing of Plant Materials

Ripe *Cassia fistula* fruits were collected from three different districts, viz., Rajshahi, Rangpur and Dhaka. The seeds were separated from the fruits, deshelled manually and washed several times with water to remove the adhesive material. Then the seeds were dried in the sun light for four consecutive days. The sun-dried

seeds were finally crushed into fine powder with the help of a grinding machine and dried in the oven at a temperature of 105 ^{0}C for an hour.

4.2.3 Extraction of Oil

The oil was extracted from powdered seed with light petroleum ether (40-60 ^{0}C) in a Soxhlet apparatus for about 24 hours. The solvent was removed by a rotary vacuum evaporator and the percentage of oil content was calculated. The crude oil thus obtained was purified in a neutral alumina column using pet. ether : diethyl ether (70:30, v/v) as the eluting solvent. The purity of the oil was checked by normal TLC. The oil was stored in glass vials and analyses were conducted as soon as possible after extraction.

4.2.4 Characterization of Oil

Physical Characteristics

The specific gravity of the oil was calculated at 35 ^{0}C with the help of a pycnometer. Refractive index of the clear oil free from water and air bubbles was determined at 30 ^{0}C using Abbe Refractometer following standard IUPAC (1979) method.

Chemical Characteristics

The acid value, percentage of free fatty acid (FFA), saponification value, saponification equivalent, unsaponifiable matters in the oil, ester value, acetyl value, Reichert-Meissl value and peroxide value were determined by the standard AOCS (1980) method. Hanus method (Bockenoogen, 1964) was followed to determine the iodine value of the oil.

4.2.5 Drying Property of oil

By this test drying oils, semidrying oils and nondrying oils are distinguished from one another, and their purities are determined. Drying property of *Cassia fistula* seed oil was determined by Elaiden Test (Das, 1989).

4.2.6 Separation of Glycerides

The whole oil was separated into mono-, di- and triglycerides by silicic acid (E. Merck, Germany, 70-230 mesh) column chromatography (Gafur, *et. al.,* 1993 a). The silicic acid was activated at 120 ^{0}C over night and again for 1 hour immediately before the column was prepared. Then the silicic acid was hydrated with 5% (w/w) water. A slurry of 25 gm of silicic acid in chloroform was poured into the column (2.2 cm i. d.). 1 gm oil was dissolved in 15 ml of chloroform and quantitatively transferred in the column. The triglyceride was eluted with 200 ml of benzene, diglyceride with 200 ml of a mixture of diethyl ether and benzene (1:9, v/v) and monoglyceride with 200 ml of diethylether. The elution was controlled at a flow rate of 1.5-2 ml / min. The amount of free fatty acid eluted with the diglyceride fraction was determined by titrating with KOH using phenolphthalein as indicator according to the method of AOCS (1980).

The elution of each fraction was monitored by micro slide thin layer chromatography (TLC) to ensure uniformity of separation of each class of glyceride during silicic acid chromatography and the eluted solvents were collected in pre-weighed flasks. The fractions thus obtained were evaporated in a rotary vacuum evaporator and were dried under reduced pressure before being weighed. The purity of glyceride classes was further checked by TLC using silica gel developed with n-hexane-diethylether (80 : 20, V/V) and visualization with

Separation of saturated and unsaturated fatty acids from about 50 gm of oil was carried out by lead-salt ether method (Sayeed *et. al.*, 1999). The oil was saponified with alcoholic caustic soda to obtain soap solution. A slight excess of lead acetate solution was added to the soap solution to form lead salts of fatty acids, which were then separated. Ether was added to the mixture of lead salts and the whole mixture was warmed and then cooled at 0 ^{0}C for 24 hours. The precipitated lead salts of saturated fatty acids so formed were separated from the solution of lead salts of unsaturated fatty acids by filtration. The lead salts of the unsaturated fatty acids were obtained by removing the ether from the ethereal solution. Each group of lead salts was suspended in water and treated with sufficient hydrochloric acid to form fatty acids and lead chlorides. The mixture was then extracted with ether to obtain the ethereal solution of the fatty acids of each group. On evaporating the ether, the fatty acids were obtained in separated groups. Finally masses of saturated and unsaturated fatty acids were obtained by weighing them separately.

4.2.9 Fatty Acid Composition of Oil

Fatty acid composition of *Cassia fistula* seed oil was analyzed as their methyl esters, which were prepared by boron-trifluoride methanol complex method (Morrison and Smith, 1964). A GCD PYE Unicam gas chromatograph equipped with a flame ionization detector was used to determine the fatty acid methyl esters. Nitrogen carrier gas was used at a flow rate of 30 ml/min. Fatty acids were separated on a 1.8 x 1/8 *i. d.* glass column packed with 6 % BDS (butanediol succinate polyesters) on solid support Anakorm ABS (100/120) mesh. Analysis was carried out at isothermal column temperature 190 ^{0}C, injector and detector temperatures for all GLC analysis were 230 ^{0}C. Gas chromatographic peaks were identified by comparison with standard methyl esters with respect to retention time

and by plotting the log of retention times against equivalent carbon length (ECL). Peaks were measured by Pye unicam electronic integrator. The percentage of each peak was calculated as the percentage of the total area of all the peaks.

4.3 Results and Discussion

The solvent extraction of *Cassia fistula* seed collected from three districts viz; Rajshahi, Rangpur and Dhaka yielded average of about 3 % oil which is appreciably higher than that of 2.04 % reported by Grindley (1946). The oil purified by a standard method was golden in color and was liquid at room temperature (25 ^{0}C). It had slightly characteristic smell. The purity of the oil was checked by normal TLC.

Characterization of Oils

A number of physical and chemical tests were carried out to determine the nature and sometimes for the identification of fats and oils. These tests may also help to evaluate the suitability of given oil or fat for a given purpose. The physical and chemical properties of an oil or fat vary between certain limits and due to the comparatively small variations, they are considered to be constants. Although the chemical constants are more important to characterize oil, yet the physical constants are also often capable of giving valuable information. Some of the more important chemical constants are iodine value, saponification value, acid value, percentage of free fatty acid, percentage of unsaponifiable matter, peroxide value etc, whereas specific gravity, refractive index etc are the important physical constants of oil.

The physico-chemical characteristics of the purified oils of *Cassia fistula* seeds collected from three different districts (Rajshahi, Rangpur and Dhaka) in

Bangladesh were determined by the conventional methods and the results were depicted in Table-4.1.

Specific Gravity

The specific gravity of fats or oils does not vary as a general rule to an extent, which makes this property useful in discriminating between one fat to another. The specific gravity of practically all fats or oils lies between 0.90 to 0.95 (Hilditch, 1949).

The specific gravities of seed oils obtained from Rajshahi and Dhaka districts were found to be 0.9259 and 0.9261 at 35 ^{0}C respectively, whereas the specific gravity of seed oils obtained from Rangpur district was estimated to be 0.9265 at 35 ^{0}C. These values are closely similar to that of sunflower oil (0.924-0.926) (Lange, 1983) and to the value 0.9241 at 30 ^{0}C reported by Kafuku Kinzo, *et. al.*, (1932) in the literature for *Cassia fistula* seed oil.

Refractive index

The refractive index of oils and fats depends to some extents on their unsaturation, and the higher refractive index represents higher unsaturation (Peach and Tracey, 1955). The oil in the sample collected from Rangpur contained highest value of refractive index (1.4753 at 30 ^{0}C) followed by Rajshahi (1.4649) and Dhaka (1.4562). These values are similar to those for olive oil (1.4657- 1.4667) (Peach and Tracey, 1955) and sunflower oil (1.4659-1.4721) (Lange, 1983). The present values are also close to the values (1.4668 at 40 ^{0}C) of *Cassia fistula* seed oil reported by Grindley (1946) and 1.4695 at 30 ^{0}C mentioned by Kafuku Kinzo, *et. al.*, (1932) .

It may be concluded from the data that the *Cassia fistula* seed oil contains fairly large amount of unsaturated fatty acids.

Acid Value and Percentage of Free Fatty Acid

Acid value is a measure of the free fatty acid present in the oils or fats and differs from the determination of the "free fatty acid" only in the interpretation and manner of expression. The assumption usually being made in a calculation that the acids have a molecular weight equal to that of oleic acid. As the liberation of free fatty acids in a natural fat or oil sample due to hydrolysis by lipase may be an important contributory factor for rancidity. A high acid value may indicate a higher tendency to become rancid. A low percentage of free fatty acid (below 1. 15%) is an indication of suitability of the oil for edible purpose (Carrol and Noble, 1957).
In the present investigation, the acid values of the *Cassia fistula* seed oil collected from Rajshahi, Dhaka and Rangpur were found to be 2.77, 3.05, and 3.41, and the percentage of free fatty acids (as oleic) were calculated to be 1.39, 1.53 and 1.71 respectively. Slightly significant difference in acid values and percentage of free fatty acids of the oils from the three samples of three districts and from the value 2.21 found in literature (Kafuku Kinzo *et. al.*,1932) is observed. This is due to the climatic and environmental factors, soil texture of the growing areas etc.

In conclusion, the present results suggest that the *Cassia fistula* seed oil might not be used as edible oil as the free fatty acid value contained in it more than 1.15 %.

Saponification Value and Saponification Equivalent

Saponification value is inversely proportional to the average molecular weight or chain length of the fatty acids present in the fat or oil. Fats and oils consisting largely of C_{18} fatty acids generally have saponification equivalent around 290.80,

indicating the presence of appreciable quantity of higher fatty acids (Ackman, 1966). The saponification values of the oils in the samples of Rajshahi, Dhaka and Rangpur were determined to be 186.37, 187.20 and 188.40 whereas saponification equivalents were calculated from saponification values to be 301.01, 299.67 and 297.77 respectively. The experimental values are in good agreement with the value 190 (Das, 1989) for sesame oil and the reported values 189.60 (Kafuku Kinzo *et. al.*,1932) and 184.90 (Grindley, 1946) for seed oil under experiment.

These comparatively low saponification values indicate the presence of higher proportion of higher fatty acids. Saponification equivalents also imply the oil contains largely of C_{18} fatty acids.

Unsaponifiable Matters

As fats and oils are encountered in commerce, they do not consist entirely of glycerides, but also contain some small percentage of substances unaffected by the saponification reaction. Unsaponifiable matters are those substances which are not saponified by alkali and which are soluble in petroleum ether or ether. In general, if a specific oil or fat has unsaponifiable matter present in excess of about 2%, there is reason to suspect adulteration. Sterols are the most common naturally occurring substances making up the unsaponifiable fraction of an oil or fat. Other substances such as vitamins A & D, unsaturated hydrocarbons such as squalene, pigments and high molecular weight monohydroxy alcohol in the case of waxes, are seen in the unsaponifiable fractions.

The *Cassia fistula* seed oil present in the samples collected from Rajshahi, Dhaka and Rangpur district contains 5.50, 5.00 and 5.30 % unsaponifiable matter respectively which are similar to those of coffee oil (5.5-6.5%) reported by

Williams (1966). In the literature, it is found that the unsaponifiable matter of the seed oil of *Cassia fistula* is 5.4% (Grindley, 1946) to not over 9.54% (Kafuku Kinzo *et. al.*, (1932). The present values are similar to that of the reported one.

It may be concluded that the *Cassia fistula* seed oil contains higher amount of unsaponifiable matters such as sterols, tocopherols, hydrocarbons, vitamins A & D etc.

Iodine Value

Iodine value gives an estimation of the degree of unsaturated fatty acids and so, of the relative amounts of unsaturated fatty acids in the trigryceride molecules of the fat or oil. The iodine values of the seed oil of *Cassia fistula* collected from Rajshahi and Dhaka districts were found to be 95.53 and 95.80 followed by Rangpur district 96.83. These values are very close to that of rape seed oil (94-100) (Das, 1989). Present experimental values are consistent with the literature values 89.47 (Kafuku Kinzo *et. al.*,1932) and 94.5 (Grindley, 1946). No significant difference in iodine values of different varieties was noticed.

It may be concluded from the result discussed above that the oil under examination contains unsaturated fatty acids to slightly higher extent.

Ester value

The ester value of an oil or fat is of value at times, and is obtained by deducting the acid value from the saponification value; it thus represents the saponification value of the neutral glycerides in 1 gm of the substance (Williams, 1966) The ester values of oils in three samples were estimated to be 183.79 (Rajshahi), 184.15 (Dhaka) and 184.99 (Rangpur) which were very close to each other.

Acetyl Value

The acetyl value indicates the number of free hydroxyl groups present in the oils or fats. Since many oils contain glycerides possessing one or more hydroxyl group not in combination with glycerin, it is possible to obtain a measurement of their presence by replacing the hydrogen of these groups by the acetyl radical. Any free alcohol such as glycerol, cholesterol, or fatty alcohol will be included if they are present (Williams, 1966). The low acetyl value indicates that the number of free hydroxyl group in the oil is low. The acetyl values of the samples of Rajshahi, Dhaka and Rangpur district were determined and found to be 18.81, 20.01 and 21.21 respectively which fell within the range of grape seed oil (10-40) (Williams ,1966).

Reichert-Meissl Value

The Reichert-Meisssl value is the measure of the volatile soluble (in water) fatty acids present in the oil or fat. The Reichert-Meissl values of the seed oils collected from Rajshahi and Dhaka district were determined and found to be 0.80 and 0.78 respectively which were very close to that of rape seed oil 0.80 (Das, 1989) while the sample of Rangpur district showed Reichert-Meissl value of 0.89 which is slightly higher than the other two.

The very low Reichert-Meissl values as obtained indicate the low content of lower volatile fatty acids; and this value is also in agreement with the low saponification value.

Peroxide Value

Fixed oils and fats absorb oxygen from the air in the autoxidation of the double bonds present in the component fatty acids. In the reaction, the oxygen adds to the

double bonds of the fatty acids forming unstable hydroperoxides. It is a measure of content of reactive oxygen, in terms of moles of peroxide or milli-equivalents of oxygen per 1 kg of oil. The oils in the samples of Rajshahi and Dhaka showed the peroxide value of 2.55 and 2.90 respectively followed by 2.71in case of the sample of Rangpur district, which were determined in normal laboratory conditions.

Table-4.1: Physical and chemical characteristics of *Cassia fistula* seed oil.

Physical and chemical characteristics	Name of the districts from where seeds were collected		
	Rajshahi	Dhaka	Rangpur
Specific gravity at 35 ^{0}C	0.9259	0.9261	0.9265
Refractive index at 30 ^{0}C	1.4649	1.4562	1.4753
Acid value	2.77	3.05	3.41
Free fatty acid (%) as oleic	1.39	1.53	1.71
Saponification value	186.37	187.20	188.40
Saponification equivalent	301.01	299.67	297.77
Unsaponifiable matters (%)	5.50	5.00	5.30
Iodine value	95.53	95.80	96.83
Ester value	183.79	184.15	184.99
Acetyl value	18.81	20.01	21.21
Reichert Meissl value	0.80	0.78	0.89
Peroxide value (m. eq. O_2/kg)	2.55	2.90	2.71

Drying Property

The oils can be classified as drying oils, semidrying oils, and nondrying oils. Amongst these, the drying oils contain high proportion of unsaturated acids, which take up oxygen from air and dry up to a hard skin. The semidrying oils contain intermediate proportion of unsaturated acids which absorb oxygen from air slowly and thickens after keeping exposed to air for some time but do not dry up. The nondrying oils contain least proportion of unsaturated acids which do not absorb oxygen, nor do they dry up to form a skin. The drying property of the seed oil of *Cassia fistula* collected from three districts was finally examined individually and found to form a treacle-like consistency after 24 hours. Hence the oil is semi-drying. According to literature (El-Sayyad *et. al.*, 1985), this oil is semi-drying.

Separation of Glycerides

The total amount of the oil was separated into mono-,di-,and triglyceride fractions by means of column chromatography and the results were presented in Table-4.2. From the results, it is seen that no appreciable change in triglyceride composition accounted for about 90 % (average) of the total weight of oil, among the three samples (Dhaka, Rajshahi and Rangpur) was noticed. Moreover the total recovery of glyceride was about 94.26 % (average) that indicated *Cassia fistula* seed oil contained a higher amount of nonglyceride than those of some common oils.

Table-4.2: Glyceride composition of *Cassia fistula* seed oil (weight %).

Name of the districts from where seeds were collected	Monoglyceride	Diglyceride	Triglyceride
Rajshahi	0.96	3.32	89.17
Dhaka	0.94	2.91	90.99
Rangpur	0.98	2.51	91.01

Fractionation of Lipids

Total extracted C*assia fistula* seed lipids were fractionated into neutral lipids, glycolipids and phospholipids by silicic acid column chromatography and the results depicted in Table-4.3 indicate that neutral lipids in all the three samples were found to be over 86 % of the total weight of the lipid. On the other hand, phospholipids were found to be highest percentage in the oil of the sample collected from Dhaka (2.70 %) and lowest percentage in the oil of the sample collected from Rangpur (1.82 %).

Table-4.3: Lipid composition of *Cassia fistula* seed oil (weight %).

Name of the districts from where seeds were collected	Neutral lipids	Glycolipids	Phospholipids
Rajshahi	86.10	4.54	2.46
Dhaka	88.03	3.92	2.70
Rangpur	89.79	3.02	1.82

Saturated and Unsaturated Fatty Acids of the Oil

The saturated and unsaturated fatty acids present in the oil were separated by lead salt ether method and the results were depicted in Table-4.4. No appreciable differences in percentage compositions of saturated fatty acids and unsaturated fatty acids of the oil in two samples collected from Rajshahi (26.15 & 68.11 %) and Dhaka (26.40 & 67.75 %) districts are found. But the fatty acid percentage compositions of the oil in the sample collected from Rangpur district (25.75 & 66.71 %) differs from those of the other two.

Table-4.4: Percentage of saturated and unsaturated fatty acids

Name of the districts from where seeds were collected	Saturated fatty acid	Unsaturated fatty acid
Rajshahi	26.15	68.11
Dhaka	26.40	67.75
Rangpur	25.75	66.71

Fatty Acid Composition of the Oil

Fatty acid analysis of the *Cassia fistula* seed oil (collected from only Rajshahi district) by GLC was carried out after transesterification of the glycerides to their methyl esters. The fatty acid composition of the oil samples was presented in Table-4.5. From the Table, it is found that *Cassia fistula* seed oil contains the highest amount of lenoleic acid (42.42, %), while oleic acid, stearic acid and palmitic acid contents are found to be 29.62 %, 14.33 %, and 11.41 % respectively. Besides these fatty acids the oil also contains small amount of myristic acid (1.44, %) and caprylic acid (0.7606, %). The GLC data also indicates

that the *Cassia fistula* seed oil contains mainly unsaturated fatty acid (72.04,%), while saturated fatty acid is found to be 27.94%. Grindley (1946) reported that saturated acids 29.1%, higher saturated acids (C_{20}- C_{24}) 1.8%, oleic acid 31.9% and linoleic acid 39% are present in *Cassia fistula* seed oil. Tsaknis *et. al.*, (1997) reported that the pumpkin seed oil contains more than 80 % of unsaturated fatty acids, of which linoleic acid and oleic acid are 40 % and 38 %, respectively while the major saturated fatty acids are palmitic acid 12.7 % and stearic acid 6 %.

From the present investigation it may be suggested that although *Cassia fistula* seed oil contains higher amount of essential fatty acid viz; linoleic acid, useful in human body, it is not used for edible purposes since it contains comparatively higher amount of saturated fatty acids.

Table-4.5: The fatty acid composition of *Cassia fistula* seed oil (weight %)

Fatty acids	Weight percent
$C_8 : 0$	0.76
$C_{14} : 0$	1.44
$C_{16} : 0$	11.41
$C_{18} : 0$	14.33
$C_{18} : 1$	29.62
$C_{18}: 2$	42.42

Chapter-5

Isolation, Purification & Characterization of Compounds from *Cassia fistula* Stem Bark

Isolation, Purification and Characterization of Compounds from *Cassia fistula* Stem Bark

5.1 Introduction

A knowledge of the biological activities and/or chemical constituents of plants is desirable, not only for the discovery of new therapeutic agents, but because such information may be of value in disclosing new sources of such economic materials as tannins, industrial oils, gums, precursors of the synthesis of complex chemical substances etc. Also, a novel chemical structure of a constituent isolated from plant sources, often prompts the chemists to a successful series of modified semisynthetic compounds e. g., atropine homatropine, reserpine syrosingopine, morphine N-allylnormorphine, which may have some medicinal or otherwise useful economic value.

The investigators primarily interested in the discovery of new biologically active plant principles would have knowledge on various approaches. Phytopharmacological approaches will consider those methods which involve, as a first step, the observation or detection of biological activity inducted by plant products. Phytochemical screening approaches will describe and evaluate methods used for the detection of phytochemical classes of compounds, examples of which are known to elicit some desirable biological response.

Natural product studies involving the search for new biological active substance present unique challenges and problems not fully appreciated by many persons having only a limited appreciation and breadth of knowledge in biological areas. Many of the problems, either enumerated or suggested by studies, can be solved if

proper attention is given to the remedies. If proper effort expanded in its own behalf, the area of natural products can assume a more important place in the scientific community to serve humanity better.

Bangladesh being situated in the monsoon area of the world, is a good treasure of indigenous plants of various families and they are grown widely in forests, jungles, hillocks and gardens. Different segments of these plants such as roots, stems, leaves, barks, flowers, seeds etc. are used by the Hakims, Kabirages and Vaidays as successful medicine of various diseases, since the ancient time not only in our country but also over the world. A systematic chemical study of these plants may lead to discovery of newer drugs having minimum or no side effects. Till now investigation is being made only on a very small portion of the vast medicinal plant population of the country that are used in indigenous medicine and a great majority of these plants remain yet to be investigation. There is a clear need for systematic research on local medicinal plants.

The present investigation was, therefore, carried out on a segment, stem bark of *Cassia fistula* that had great medicinal significance, to expose it to the modern technology.

5.2 Materials and Methods

5.2.1 Materials

All the reagents and chemicals used for the present work were purchased from BDH (England), FLUKA (Switzerland) and E. Merck (Germany). Solvents used during the work were purified by distillation at the boiling point of the respective solvents. The IR spectra were recorded on a PERKIN ELMER 1600 FTIR instrument. A Reichart micro melting point apparatus was used for recording the

melting points. ^{1}H NMR (400 and 500 MHz) spectra and ^{13}C NMR (100 and 125 MHz) spectra were recorded with varian NMR Spectrometers in $CDCl_3$ using TMS as internal standard. The mass spectra were registered at low resolution in a mass spectrometer.

5.2.2 Collection and Processing of Plant Materials

Stem bark of matured *Cassia fistula* was collected from Rajshahi college campus, Rajshahi. Adhering dusts and particles were removed from the plant barks by washing with water and clean barks were dried in the sunlight. Then the barks were cut into very small thin pieces and dried in the sunlight for two days and sun dried materials were kept in an electric oven at 50 ^{0}C for about 72 hours. The dried materials were taken out from the oven and milled to yield a fine powder. The powdered materials were 1.30 kg and stored in an airtight container.

5.2.3 Extraction of Plant Materials Using Solvents of Various Polarities

Extraction was carried out at room temperature with gentle stirring for nine days (three times within this period) from the dried powdered material (1.30 kg) using ethyl acetate as extracting solvent. The resultant extracts were combined and the combined extract was filtered and concentrated under a vacuum to obtain semi-solid mass. The semi-solid mass thus obtained was then washed with light petroleum ether (b.p.40-60 ^{0}C) to yield petroleum ether extract. Residues left after extraction with ethyl acetate were dried in air and again extracted with methanol in the same procedure as depicted above. The percentage of yields of different solvent extracts of stem bark is depicted in Table-6.1 (Chapter-6). A schematic diagram on overall extraction process is shown in Figure-5.1.

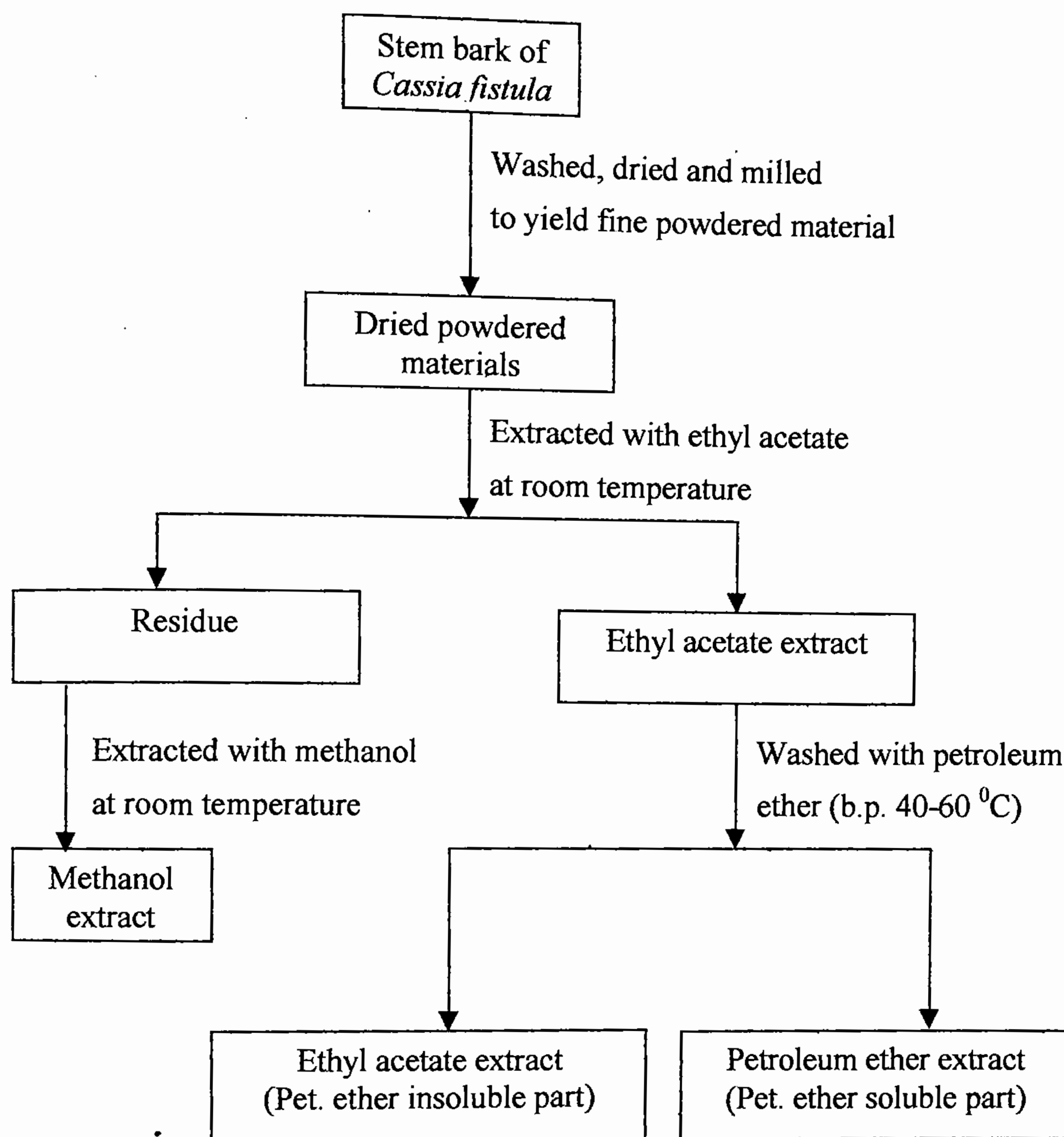


Figure-5.1: Extraction of plant materials using solvents of various polarities.

5.2.4 Isolation and Purification of Compounds from the Crude Ethyl Acetate Extract Using Various methods

Column Chromatography of the Crude Ethyl Acetate Extract (Beckett and Stenlake, 1986).

Column chromatography of the semi-solid product obtained from ethyl acetate extract was carried out to separate the individual components. The crude ethyl acetate extract (6.80 gm) was mixed well with sufficient silica gel (70-230 mesh) and dried in a vacuum dissiccator over P_2O_5. This crude mixture was poured slowly into the column previously packed by silica gel (70-230 mesh) and was covered with a layer of cotton. The column was eluted using different solvent systems of increasing polarities. Each fraction was collected in 100-ml beaker and the solvent from the fraction was removed under reduced pressure. The results are depicted in Table-5.1.

Table-5.1: Column chromatography of crude ethyl acetate extract over silica gel (70-230 mesh)

Eluents	Amount of solvent	Fraction No.
100 % n-hexane	200	1-2
25 % pet. ether(40-60 ^{0}C) in n-hexane	100	3
50 % pet. ether(40-60 ^{0}C) in n-hexane	100	4
100 % pet. ether(40-60 ^{0}C)	200	5-6
2.5 % ethyl acetate in pet. ether(40-60 ^{0}C)	100	7
5 % ethyl acetate in pet. ether(40-60 ^{0}C)	100	8
7.5 % ethyl acetate in pet. ether(40-60 ^{0}C)	100	9
10 % ethyl acetate in pet. ether(40-60 ^{0}C)	100	10
12.5 % ethyl acetate in pet. ether(40-60^{0}C)	100	11
15 % ethyl acetate in pet. ether(40-60 ^{0}C)	100	12
20 % ethyl acetate in pet. ether(40-60 ^{0}C)	200	13-14
25 % ethyl acetate in pet. ether(40-60 ^{0}C)	100	15
30 % ethyl acetate in pet. ether(40-60 ^{0}C)	200	16-17
40 % ethyl acetate in pet. ether(40-60 ^{0}C)	100	18
50 % ethyl acetate in pet. ether(40-60 ^{0}C)	100	19
60 % ethyl acetate in pet. ether(40-60 ^{0}C)	200	20-21
70 % ethyl acetate in pet. ether(40-60 ^{0}C)	100	22
80 % ethyl acetate in pet. ether(40-60 ^{0}C)	200	23-24
90 % ethyl acetate in pet. ether(40-60 ^{0}C)	100	25
100 % ethyl acetate	300	26-28
5 % methanol in ethyl acetate	200	29-30
10 % methanol in ethyl acetate	100	31
20 % methanol in ethyl acetate	100	32
35 % methanol in ethyl acetate	200	33-34
50 % methanol in ethyl acetate	100	35
75 % methanol in ethyl acetate	100	36
100 % methanol	200	37-38

Analysis of Eluted Fractions

Each of the fractions was analyzed by thin layer chromatography (Egon and Stahl, 1969; Randerath, 1966) using silica gel (GF_{254}) as stationary phase. Different types of solvent systems were used for resolution of the compounds on the TLC plates. Depending on the TLC behavior these were classified on the following groups:

Groups	Fraction numbers
Group-A	1-6
Group-B	7-12
Group-C	13-19
Group-D	20-24
Group-E	25-28
Group-F	29-32
Group-G	33-38

Preparative TLC of Various Groups (Egon and Stahl, 1969; Randerath, 1966)

Groups B, C, D and E were subjected to preparative TLC using different solvent systems as mobile phase. Silica gel (GF_{254}) was used as stationary phase. Two compounds designated as CBC-1 (21 mg) and CBC-2 (26 mg) were isolated from group-B and one compound designated as CBC-3 (33 mg) was isolated from group-C. Group-D was discarded due to absence of any clear spot on TLC plate. Group-E contained a mixture of compounds, which was difficult to separate by PTLC. So further attempt was not taken with this group. Although other fractions show a number of spots but the amount of each fraction was small relative to the number of compounds observed by TLC examination. So these fractions were not analyzed. The purity of isolated compounds was checked by TLC under UV-light

and in iodine chamber using various solvent systems (Table-5.2). Moreover, the solubility of the compounds was examined using different solvents shown in Table-5.3.

Table-5.2: Developing solvent systems with R_f values of the pure compounds.

Sample code name	Solvent system	R_f value
CBC-1	Pet. ether : Diethyl ether (4:1)	0.331
	n-Hexane : Ethyl acetate (8 : 1)	0.820
CBC-2	Pet. ether : Ethyl acetate (2 : 1)	0.593
CBC-3	n-Hexane : Ethyl acetate (7 : 1)	0.593
	Pet. ether : Chloroform(10 : 1)	0.610

Table-5.3 : Solubility behavior of the pure compounds.

Solvent	Solubility		
	CBC-1	CBC-2	CBC-3
Petroleum ether (40-60 ^{0}C)	Insoluble	Insoluble	Sparingly soluble
Acetone	Soluble	Soluble	Soluble
Ethyl acetate	Soluble	Soluble	Soluble
Chloroform	Soluble	Soluble	Soluble
Methanol	Insoluble	Insoluble	Insoluble
Water	Insoluble	Insoluble	Insoluble

5.2.5: Characteristics of Isolated Pure Compounds

To characterize each of the isolated compounds, physical and chemical properties as well as spectral data were studied. The physical properties such as physical

form, melting point, R_f value and solubility were determined. Chemical properties mainly the test for nitrogen, sulfur, halogen (as elemental test), aldehydes, ketones, carboxylic acid, carbohydrate and unsaturation (Vogel, 1956) as well as test for alkaloids (Peach and Tracey, 1955), steroids or terpenoids (Finar,1983) and for alcohols (Feigl, 1966) were carried out. Among spectroscopic methods, IR, ^{1}H-NMR, ^{13}C-NMR and Mass spectral data were recorded.

Characteristics of Compound CBC-1

Physical form: Needle shaped crystals

Melting point: 133^{0}-135 ^{0}C

R_f value: Shown in Table-5.2

Solubility: Shown in Table-5.3

It gave pink color on TLC with vanillin sulfuric acid spray reagent on heating the plates at 110 ^{0}C until coloration took place. It did not give positive test for alkaloids and hydrocarbons. It gave positive Salkowski and Liebermann-Burchard test for steroids or terpenoids.

^{1}H-NMR (Figure-5.2) and ^{13}C-NMR (Figure-5.3) spectral data of compound CBC-1 were recorded in $CDCl_3$ using TMS as the internal standard which were shown in Table-5.4 and Table-5.5 respectively.

Table-5.4: ^{1}H-NMR (500 MHz) spectral data of compound CBC-1.

Position of protons	Chemical shift (δ value in ppm, J in H_Z)
H-3	3.53 (1 H, m,)
H-6	5.36 (1 H, d, J=4.46)
H-18	0.68 (3 H, s, CH_3)
H-19	0.85 (3 H, s, CH_3)
H-21	1.01 (3 H, d, J=6.5, CH_3)
H-26	0.92 (3 H, d, J=6.42, CH_3)
H-27	0.87 (3 H, d, J=6.42, CH_3)
H-29	0.90 (3 H, t, J=6.50, CH_3)

Table-5.5: ^{13}C-NMR (125 MHz) spectral data of compound CBC-1.

Carbon number	Chemical shift (δ value in ppm)	Carbon number	Chemical shift (δ value in ppm)
C-1	37.3	C-16	28.2
C-2	31.7	C-17	56.1
C-3	72.5	C-18	12.0
C-4	42.4	C-19	19.4
C-5	140.8	C-20	36.1
C-6	121.7	C-21	19.3
C-7	31.9	C-22	34.0
C-8	31.9	C-23	29.2
C-9	50.1	C-24	50.1
C-10	36.4	C-25	26.1
C-11	21.1	C-26	19.0
C-12	39.8	C-27	19.4
C-13	42.3	C-28	23.1
C-14	56.8	C-29	11.9
C-15	24.3		

Characteristics of Compound CBC-2

Physical form: White crystalline solid.

Melting point: 295^0-$297\ ^0C$

R_f value: Shown in Table-5.2

Solubility: Shown in Table-5.3

The compound CBC-2 gave positive test for steroids or terpenoids with Salkowski and Liebermann-Burchard reactions and negative test for alkaloid and carbohydrate. Brown colour of bromine in CCl_4 discharged by the compound indicated the presence of unsaturation. The solubility of the compound in both NaOH and $NaHCO_3$ (carbondioxide escaped as bubble) indicated the presence of carboxylic acid in the compound.

IR ν_{max} cm^{-1}: 3624, 3074, 1705, 1641 and 884 cm^{-1} (Figure-5.4).

^{1}H-NMR (Figure-5.5) spectral data of the compound CBC-2 were recorded in $CDCl_3$ using TMS as the internal standard which were given in Table-5.6.

Table-5.6: ^{1}H-NMR (500 MHz) spectral data of compound CBC-2.

Position of protons	Chemical shift (δ value in ppm, J in Hz)
H-3	3.13 (1 H, m)
H-19	2.94 (1 H, dt, J=10.5, 4.2)
H-29	4.543 and 4.675 (1 H each, d, J=2.0)
H-23	0.963 (3 H, s, CH_3)
H-24	0.823 (3 H, s, CH_3)
H-25	0.862 (3 H, s, CH_3)
H-26	0.987 (3H, s, CH_3)
H-27	0.916 (3H, s, CH_3)
H-30	1.634 (3 H, s, CH_3)

Characteristics of Compound CBC-3

Physical form: White needle shaped crystals.

Melting point: 212-215 ^{0}C

R_f value: Shown in Table-5.2

Solubility: Shown in Table-5.3

The compound CBC-3 gave positive test for steroids or terpenoids with Salkowski and Liebermann-Burchard reactions. It gave negative test for aldehydes, alkaloid and carbohydrate. Brown color of bromine in CCl_4 discharged by the compound CBC-2 indicated the presence of unsaturation.

IR v_{max} cm^{-1}: 3352.90, 1639 and 881.5 cm^{-1} (Figure-5.6).

^{1}H-NMR (Figure-5.7) and ^{13}C-NMR (Figure-5.8) spectral data of compound CBC-2 were recorded in $CDCl_3$ using TMS as the internal standard which were given in Table-5.7 and Table-5.8 respectively.

Table-5.7: ^{1}H-NMR (400 MHz) spectral data of compound CBC-3.

Position of protons	Chemical shift (δ value in ppm, J in H_Z)
H-3	3.20 (1 H, dd, J=11.2, 5.1)
H-19	2.39 (1 H, m)
H-29	4.58 (1 H, m), 4.70 (1 H, d, J=2.4)
H-23	0.95 (3 H, s, CH_3)
H-24	0.77 (3 H, s, CH_3)
H-25	0.84 (3 H, s, CH_3)
H-26	1.04 (3H, s, CH_3)
H-27	0.97 (3H, s, CH_3)
H-28	0.80 (3 H, s, CH_3)
H-30	1.69 (3 H, s, CH_3)

Table-5.8: ^{13}C-NMR (100 MHz) spectral data of compound CBC-3.

Carbon number	Chemical shift (δ value in ppm)	Carbon number	Chemical shift (δ value in ppm)
C-1	38.95	C-16	35.82
C-2	27.66	C-17	43.27
C-3	79.24	C-18	48.55
C-4	39.09	C-19	48.22
C-5	55.54	C-20	151.19
C-6	18.56	C-21	30.09
C-7	34.53	C-22	40.24
C-8	41.08	C-23	28.22
C-9	50.68	C-24	15.59
C-10	37.41	C-25	16.34
C-11	21.17	C-26	16.21
C-12	25.39	C-27	14.78
C-13	38.30	C-28	18.23
C-14	43.07	C-29	109.54
C-15	27.69	C-30	19.54

Mass Spectrum:

m/z value (rel. %): 426 [M^+] (81 %), 408 (16 %), 218 (56 %), 208 (86 %), 203 (55 %) and 189 (100 %).

5.3 Results and Discussion

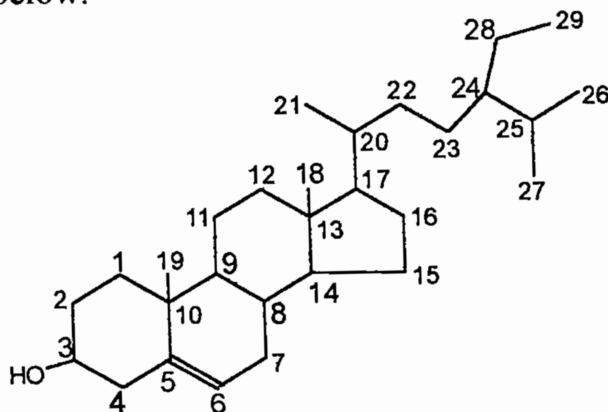
Characteristics of Compound CBC-1

The crystalline compound CBC-1 isolated from crude ethyl acetate extract of *Cassia fistula* bark was found to be homogeneous on TLC plates using various solvent systems and gave positive test for alcohol and steroid with Salkowski and Liebermann-Burchard reactions and negative test for hydrocarbons and for alkaloid with Dragendorff's reagent. Yellow color developed with tetranitromethane showed the presence of unsaturation. From the positive tests for steroid and alcoholic function shown by the compound CBC-1, it was assumed to be a sterol. The melting point of this compound (133-135 ^{0}C) is in good agreement with the melting point of β-sitosterol in the literature (Ching-Kuo Leea *et. al.*,2001)

In ^{1}H-NMR spectrum (Figure-5.2 & Table-5.4), the compound CBC-1 showed two tertiary methyl proton peaks at δ 0.68 (3H, s, H-18) and δ 0.85 (3H, s, H-19), three secondary methyl proton peaks at δ 0.92 (3H, d, J=6.42 Hz, H-26), δ 0.87 (3H, d, J=6.42 Hz, H-27) and δ 1.01 (3H, d, J=6.5 Hz, H-21) and one primary methyl proton peak at δ 0.90 (3H, t, J=6.5 Hz, H-29). The spectrum also exhibited a broad doublet at δ 5.36 (1H, d, J=4.6 Hz) attributed to be a double bonded proton typical for H-6 and multiplet at 3.53 (1H, m) integrated for one proton which might be H-3 of a steroidal skeleton. In addition, other signals appeared between δ 0.9~2.4 were due to the methylene and methine protons.

Moreover, ^{13}C-NMR spectral data of compound CBC-1 (Figure-5.3 & Table-5.5) were quite similar to those of authentic β-sitosterol cited in the literature. Based on the spectral data as well as chemical evidence in corporation with physical

properties, the compound was finally identified and confirmed as β-sitosterol. The structure is depicted below:



β-Sitosterol (CBC-1)

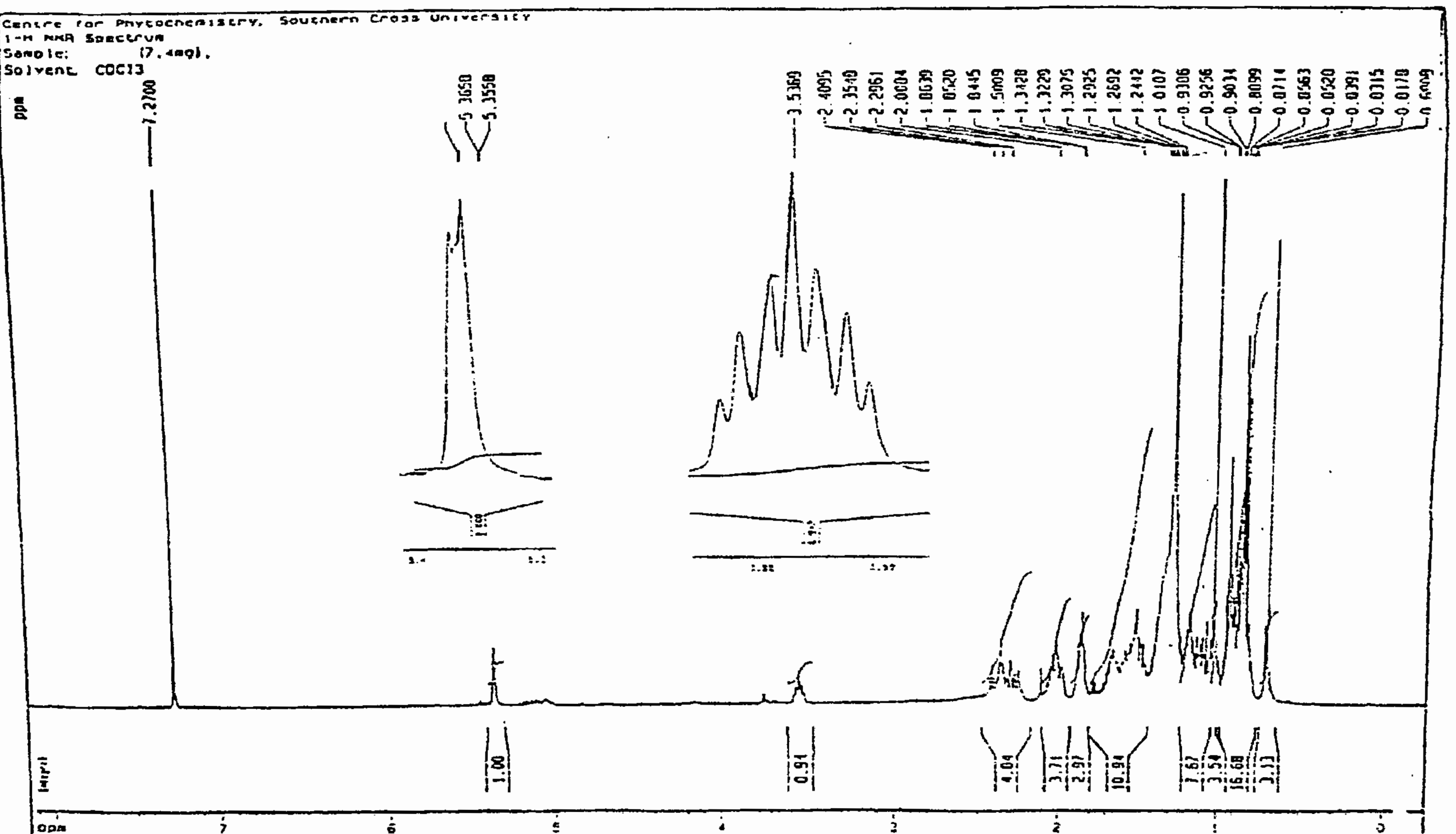


Figure-5.2: ^{1}H-NMR spectrum of compound CBC-1

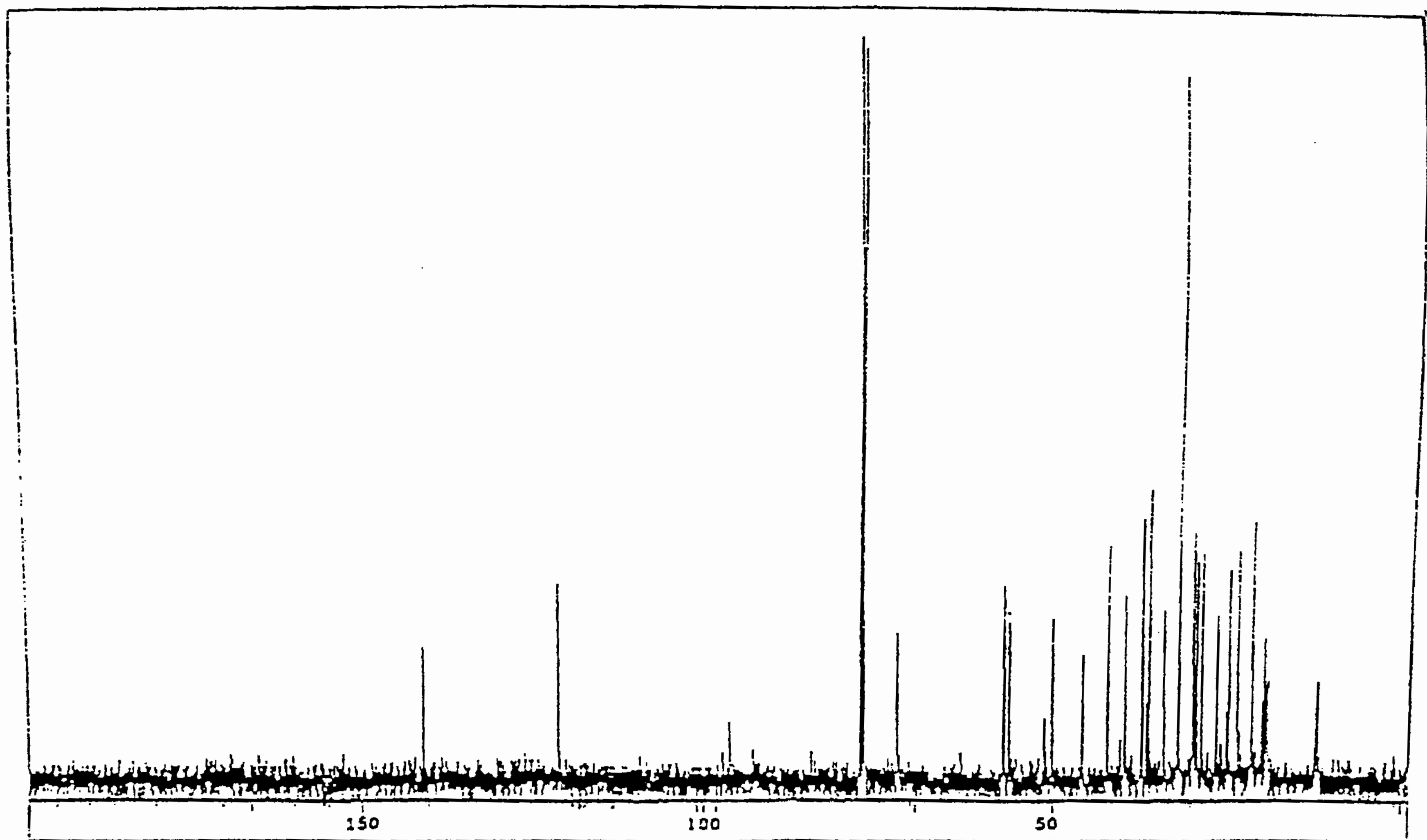


Figure-5.3: ^{13}C-NMR spectrum of the compound CBC-1

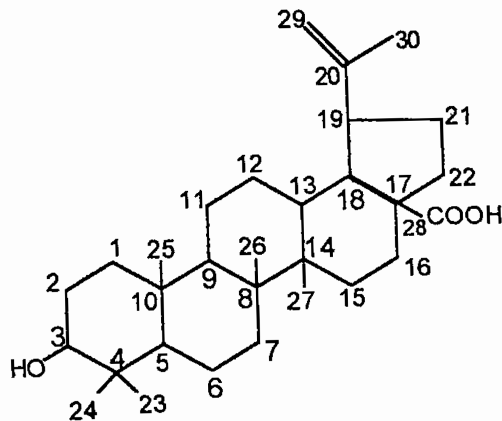
Characteristics of Compound CBC-2

The ethyl acetate extract of *Cassia fistula* stem bark after chromatography over silica gel yielded a pure compound CBC-2 as a white crystalline form having melting point 295-297 ^{0}C. It showed single spot on TLC plate after spraying with vanillin-sulphuric acid spray reagent and heating. It gave negative test for alkaloid and carbohydrates but the test for unsaturation was positive when treated with bromine in carbontetrachloride solution. The solubility of the compound in both sodium hydroxide and sodium bicarbonate solution indicated the presence of carboxyl functional group in the compound. Positive Salkowski and Liebermann-Burchard coloration tests for the compound CBC-2, suggested it to be a steroid or a triterpenoid.

The IR spectrum (Figure-5.4) of the compound exhibited strong absorption bands at 3624 and 1705 cm^{-1} which might be due to hydroxyl group (–OH) and a carbonyl in carboxyl function respectively and also stretching at 1641 cm^{-1} and 884 cm^{-1} due to an exomethylene group.

The ^{1}H-NMR spectrum (Figure-5.5 & Table-5.6) also supported the presence of exomethylene protons at δ 4.543 and δ 4.675 (1 H each, d, J=2.0) for H-29. The spectrum also showed a vinylic methyl protons as singlet at δ 1.634 for H-30 and five tertiary methyls at δ0.823, δ0.862, δ0.916, δ0.963 and δ0.987 for H-24, H-25, H-27, H-23 and H-26 respectively. A carbinylic proton at C-3 was assigned by the presence of characteristic signal at δ 3.13 (1 H, m) which was highly de-shielded due to the OH substituent at C-3. A single proton at C-19 was assigned by the double triplet at δ 2.94. All the proton peaks in ^{1}H-NMR spectrum of the compound CBC-2 are in good agreement with those of betulinic acid (Herz *et. al.*, 1972).

On the basis of spectral data coupled with physical and chemical evidences, the structure of the compound CBC-2 was thus identified as Bet-20 (29)-en-3-ol-28-oic acid. The structure is given below:



Bet-20 (29)-en-3-ol-28-oic acid (CBC-2)

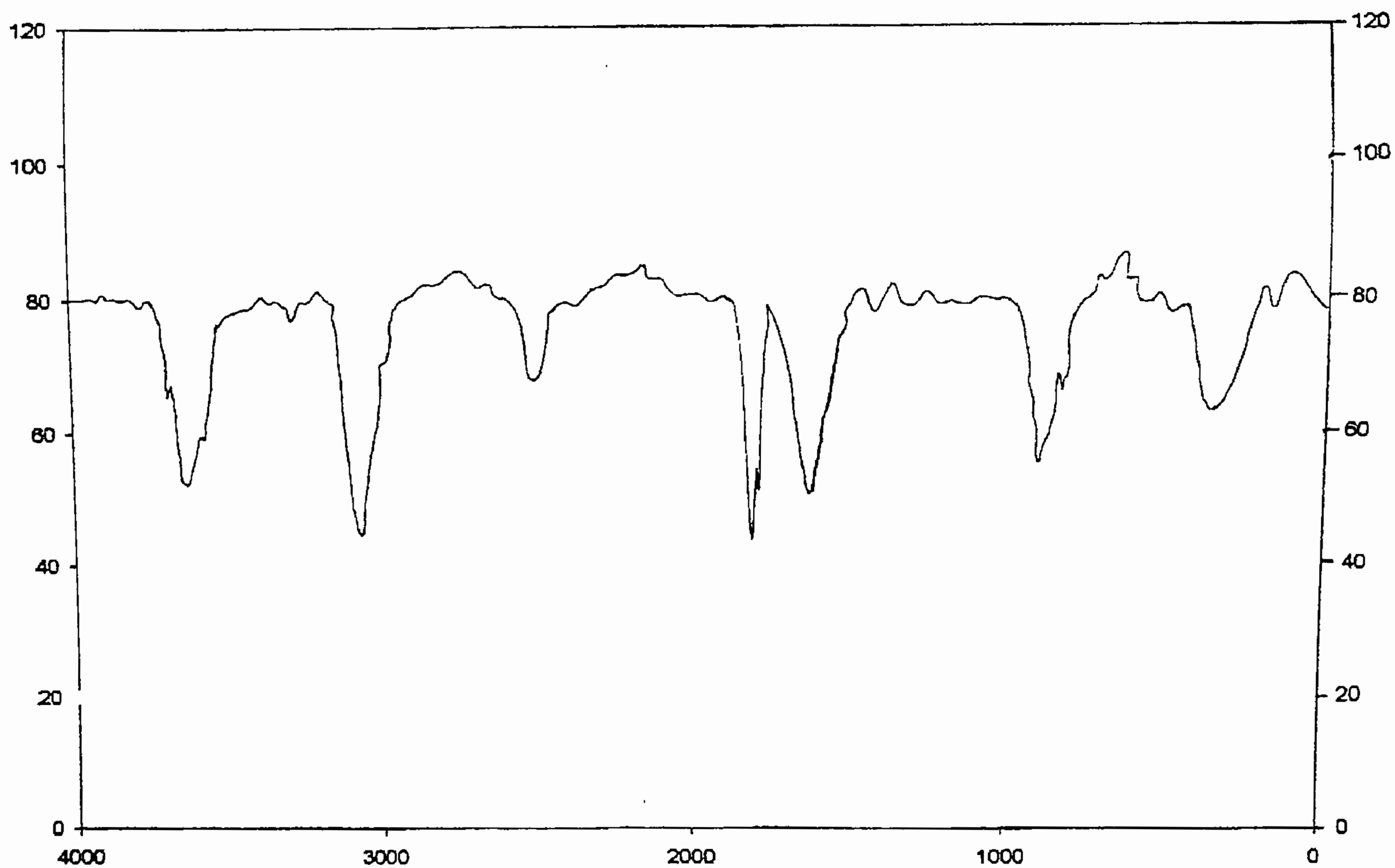


Figure-5.4: IR spectrum of compound CBC-2

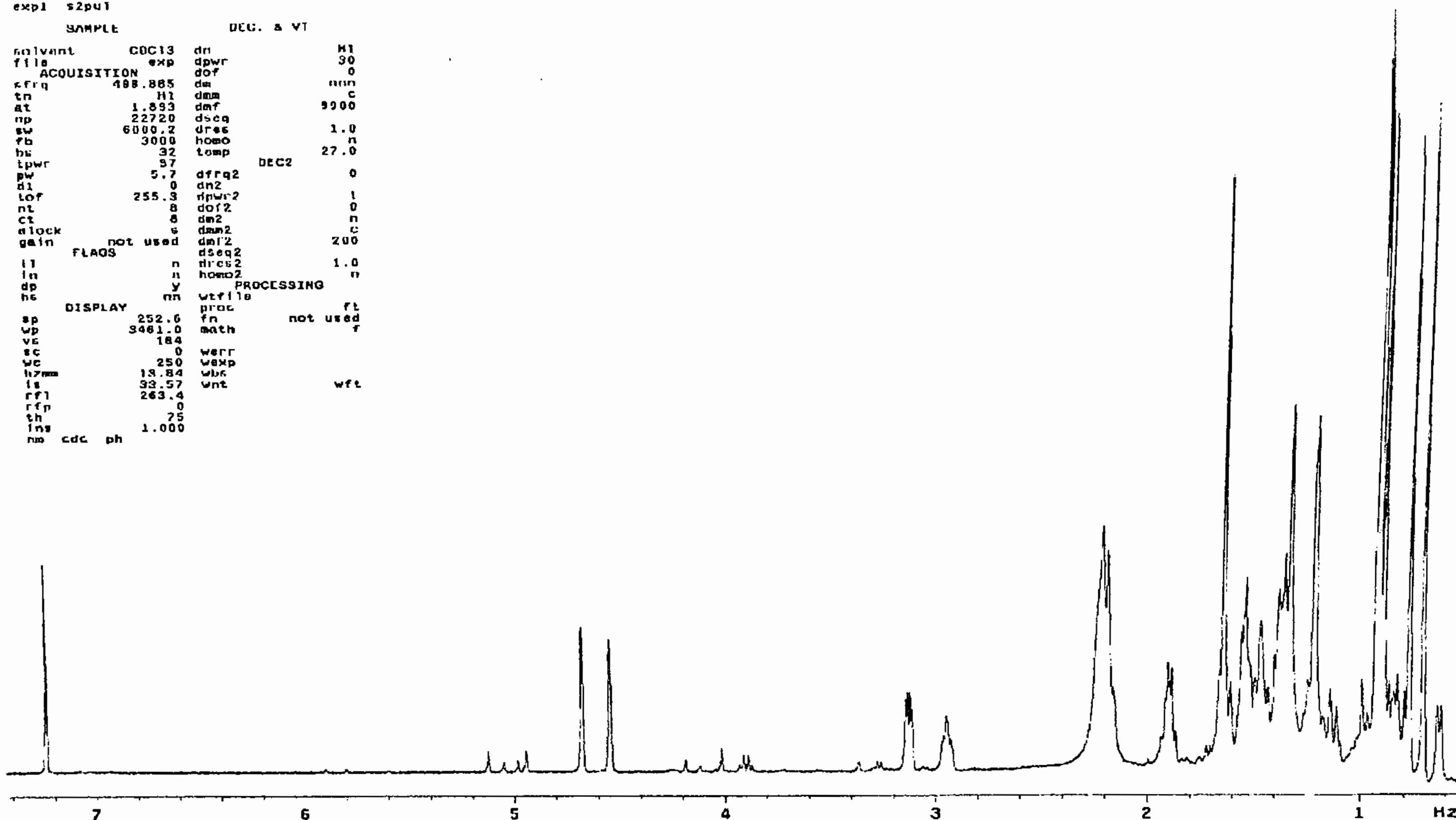


Figure-5.5: ^{1}H-NMR spectrum of compound CBC-2

Characteristics of Compound CBC-3

The ethyl acetate extract of *Cassia fistula* stem bark after chromatography over silica gel yielded a white crystalline compound CBC-3 having melting point 212-215 ^{0}C. It showed a single spot on TLC plate using various solvent systems as pink color when sprayed with vanillin-sulphuric acid spray reagent. It gave negative test for aldehydes, alkaloid and carbohydrate but the test for unsaturation was positive when treated with bromine in carbontetrachloride solution. Positive Salkowski and Liebermann-Burchard coloration tests by the compound CBC-3, suggested it to be a steroid or a triterpenoid.

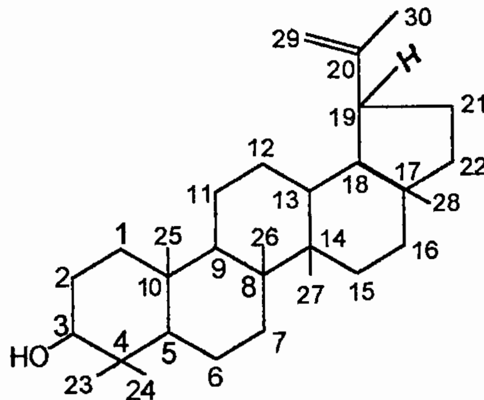
The IR spectrum (Figure-5.6) of the compound showed strong absorption bands at 3352.90 cm^{-1} might be due to hydroxyl group (–OH) and at 1639 cm^{-1} and 881.5 cm^{-1}, which were typical for an exomethylene group.

The ^{1}H-NMR spectrum (Figure-5.7 & Table-5.7) also supported the presence of exomethylene protons at δ 4.58 (1 H, m) and δ 4.70 (1 H, d, J=2.4) for H-29. The spectrum also indicated a vinylic methyl protons as singlet at δ 1.69 for H-30 and six tertiary methyls at δ 0.77, δ 0.80, δ 0.84, δ 0.95, δ 0.97 and δ1.04 for H-24, H-28, H-25, H-23, H-27 and H-26 respectively. Other important signals exhibited by the compound CBC-3 in ^{1}H-NMR spectrum were a double doublet at δ 3.20 and a multiplet at δ 2.39 attributed to H-3 and H-19 respectively. The proton H-3, is due to the OH substituent of the steroidal ring and the peak is assigned for CH-OH. All the proton peaks found in ^{1}H-NMR spectrum of the compound CBC-3 are in consistent with those of lupeol (Kahlos and Hiltunen, 1989).

Moreover, ^{13}C-NMR spectrum of compound CBC-3 (Figure-5.8 & Table-5.8) showed a total of 30 carbons. Among these, the exomethylene carbon appeared at δ109.54, the quaternary carbon attached to be exomethylene at δ151.19 and the oxygenated methine at δ79.24. The chemical shifts of all the carbons were in close agreement with those of lupeol (Kahlos and Hiltunen, 1989).

Again, the mass spectrum (Figure-5.9) of the compound revealed the highest ion peak at m/z 426, which suggested the molecular weight 426 corresponding to the molecular formula $C_{30}H_{50}O$, a number of prominent peaks at m/z 408 (16 %), 218 (56%), 208 (86 %), 203 (55 %), and 189 (100 %).

On the basis of physical and chemical characteristics coupled with spectral data, the compound CBC-3 was finally identified as lupeol. The structure is given below:



Lupeol (CBC-3)

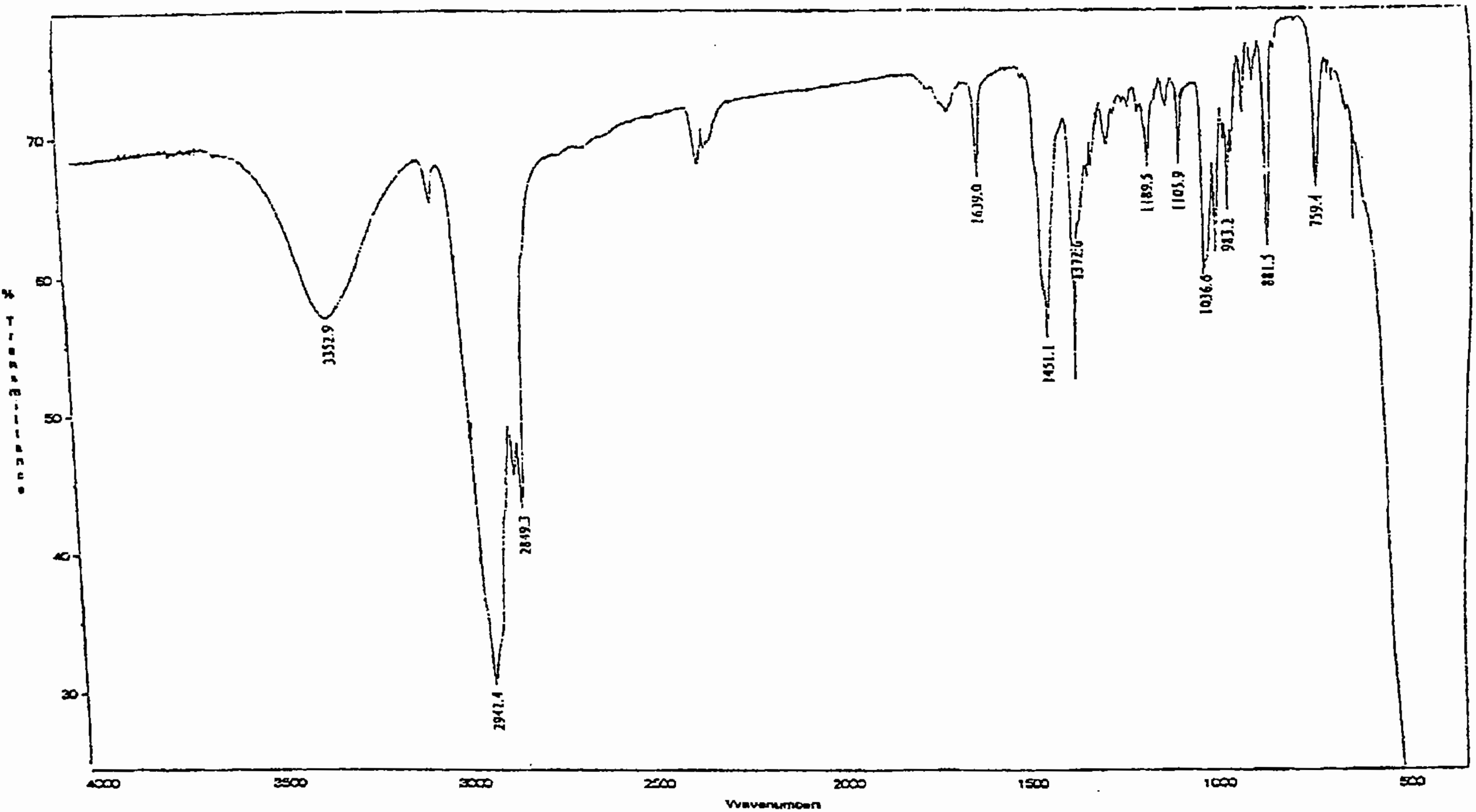


Figure-5.6: IR spectrum of compound CBC-3

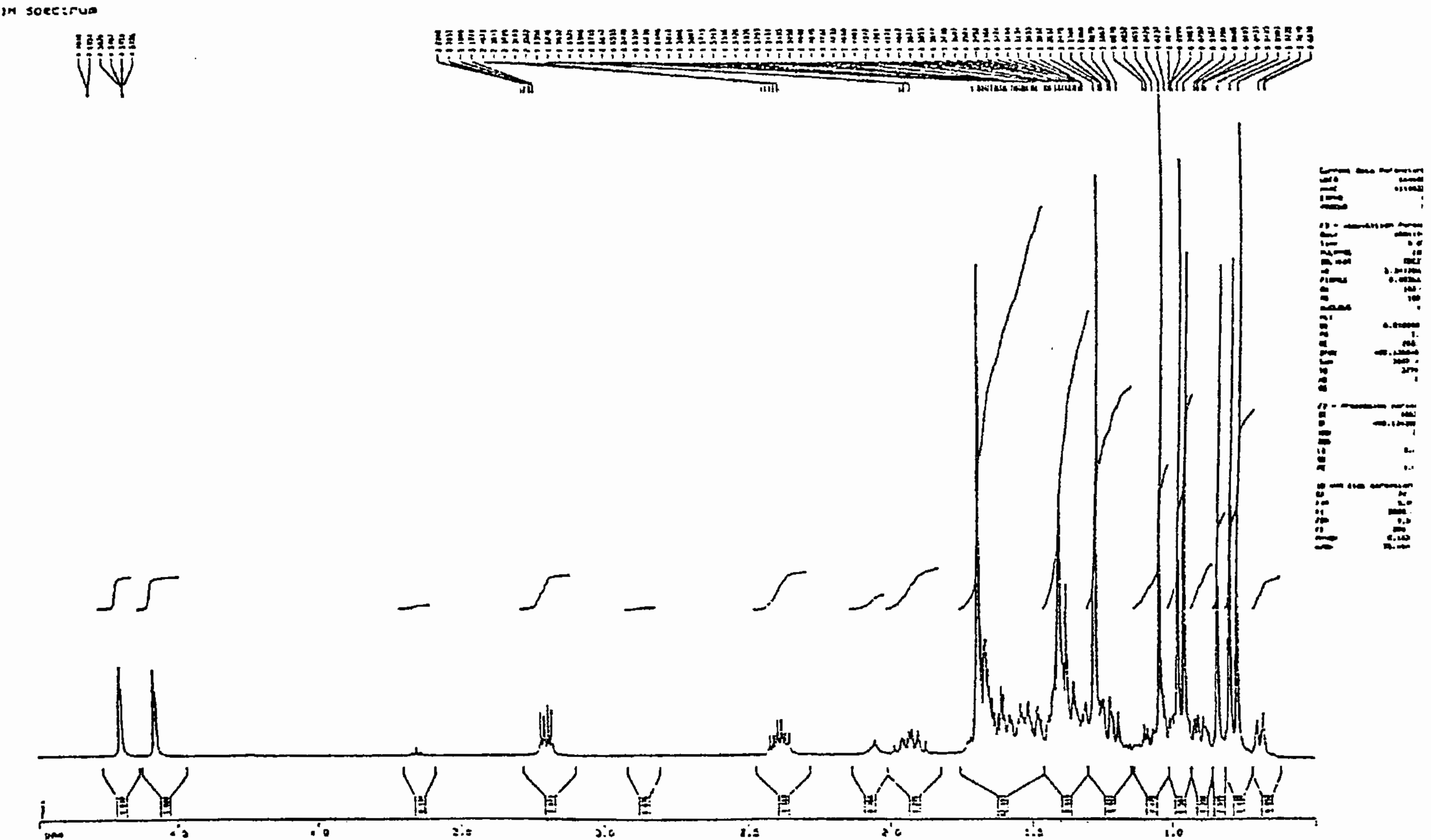


Figure-5.7: ^{1}H-NMR spectrum of compound CBC-3

Figure-5.8: ^{13}C-NMR spectrum of compound CBC-3

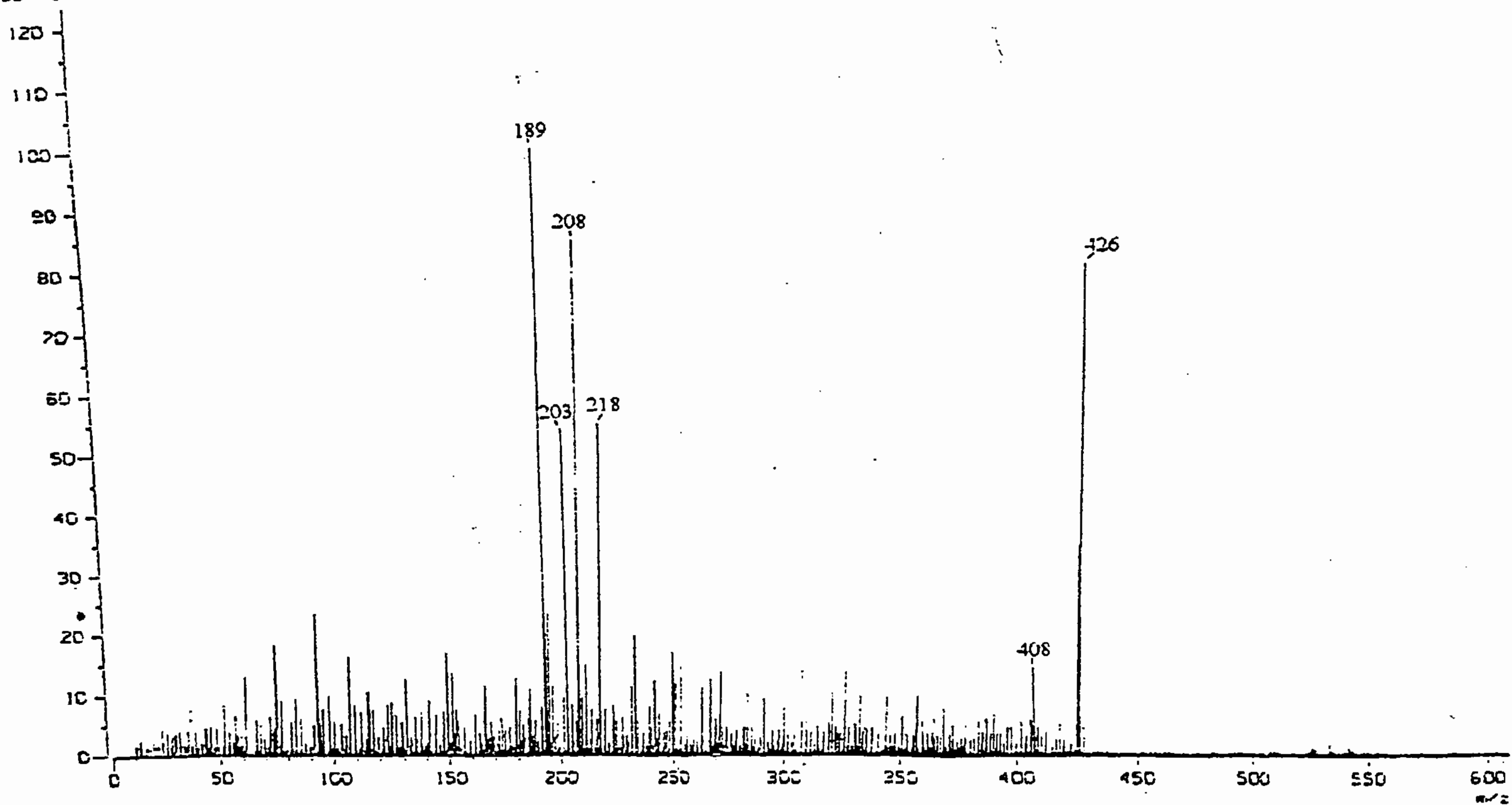


Figure-5.9: Mass spectrum of compound CBC-3

Chapter-6

Biological Investigations on *Cassia fistula*

Biological Investigations on *Cassia fistula*

6.1 Micromiological Investigation

6.1.1 Introduction

The world of microbiology is a world of very minute organism, which includes the study of living organisms of microscopic size namely bacteria, fungi, algae, protozoa and viruses. This world is a biological universe where human beings have begun to explore only recently. Human eyes fist saw microorganisms barely 500 years ago and serious study began perhaps 200 years ago. Microorganisms are closely associated with the health and welfare of human beings. Most microorganisms are free living and perform useful activities that benefit animal and plant life. For example, microorganisms are involved in making yogurt, cheese, and wine, in the production of penicillin, interferon and alcohol and in the processing of domestic and industrial wastes. Microorganisms are used as tool to explore fundamental life process especially in the field of Molecular Biology.

Many microorganisms can cause several diseases and now, in this world of modern science, man can face any challenge against any disease. But in spite of the tremendous advancement of medical science and technology, diseases are the leading health problem particularly in the under privileged population in the remote rural areas in the developing countries. In Bangladesh, a very poor country with poor hygiene, diarrhoea, cholerae, typhoid, malaria, dyptheria etc. are major causes of morbidity and mortality. Recent information tells that diarrhoea, cholerae, typhoid, and malaria have been spreading alarmingly. Diarrhoea is the major killer disease particularly in children during the last quarter of the 20th century in Bangladesh as well as in the whole world. Every year diarrhoeal

diseases claim 5 to 10 millions lives of children out of 3 to 5 billions incidence in Asia, Africa and Latin America (Walsh and Warren, 1979). Among this about 200,000 children died in diarrhoea annually only in Bangladesh.

Shigellosis is predominant among all diarrhoeal diseases and in Bangladesh alone about 80 to 85% of bacillary dysentery is due to *Shigella* species (Walsh and Warren, 1979). Generally four strains of *Shigella* are responsible for bacillary dysentery which are *Shigella dysenteriae*, *Shigella flexneriae*, *Shigella boydii* and *Shigella Sonnei*. According to a report of clinical research centre, ICDDR, B 13.7 % of all diarrhoeal diseases in 1990 were associated with *Shigella* species and the corresponding figure for 1991 was 16.1 % with a mortality rate of 12.7 % (ICDDR, B, 1991).

Plant products have been used from time immemorial for the treatment of diseases. Treatment of diseases with extracts of seeds, roots, rhizomes, barks and leaves of plant has been a common phenomenon in our country. Most of the medicines used by millions of patients in the different parts of the world come directly or indirectly from plant sources. So, a desperate need to search out new antibacterial and anti-fungal agents from plants to relief the killer diseases in perspective of Bangladesh.

It has been expected that the present work on anti-microbial screening of the plant materials of *Cassia fistula* will lead to the scientists that continue work may have clinical success concerning the killer diseases.

6.1.2 Materials and Methods

6.1.2.1 Materials

The organisms used in present studies were collected from the Department of Pharmacy, University of Rajshahi, the pure cultures of which were previously

collected from the Institute of Food & Nutrition, University of Dhaka, and also from ICDDR, B. All solvents used for this study were redistilled. Other chemicals, including culture media used were of analytical grade.

6.1.2.2 Processing of Plant Materials

Fruits, leaves and stem bark of *Cassia fistula* were collected from Rajshahi college campus, Rajshahi. Processing of stem bark was described in chapter-5. Seeds and pods were separated from the fruits. Leaves, seeds and pods were individually washed several times with water to remove adhering materials and dried in the sunlight for two days. The sun dried plant materials were dried again in an electric oven at 50 ^{0}C for about 72 hours and finally powdered separately with the help of a grinding machine.

6.1.2. 3 Extraction of Plant Materials Using Solvents of Various Polarities

Extraction was carried out with petroleum ether, ethyl acetate and methanol as in the same procedure of the extraction of stem bark described in chapter-5. Each of the extracts was concentrated at low temperature and reduced pressure using a rotary evaporator. Percentages of yield of different solvent extracts are given in Table-6.1.

Table-6.1: Percentage of yield of different solvent extracts of the plant *Cassia fistula*.

Plant material	Weight of powdered plant material (gm)	Weight of petroleum ether extract (gm)	Weight of ethyl acetate extract (gm)	Weight of methanol extract (gm)	Percentage of yield (%)		
					Petroleum ether extract	Ethyl acetate extract	Methanol extract
Stem bark	1300	45.578	66.208	49.53	3.506	5.092	3.810
Leaves	200	4.089	3.092	5.054	2.044	1.546	2.527
Pods	200	2.987	3.921	6.906	1.493	1.960	3.453
Seeds	200	4.061	5.715	5.023	2.030	2.857	2.511

6.1.2.4 Antibacterial and Antifungal Screening

The antibacterial and antifungal activities of crude petroleum ether, ethyl acetate and methanol extracts as well as two pure compounds, CBC-2 [Bet-20 (29)-en-3-ol-28-oic acid] and CBC-3 (Lupeol) isolated from ethyl acetate extract of *Cassia fistula* stem bark were investigated against 14 pathogenic bacteria and 6 fungi respectively. On the other hand, three lectins, CSL-1, CSL-2 & CSL-3 purified from *Cassia fistula* seeds were tested against only 14 pathogenic bacteria. Nutrient agar medium was used for determining antibacterial activity whereas potato dextrose agar (PDA) medium was selected for antifungal screening. Standard antibiotic disc of kanamycin (30 μg / disc) for antibacterial activity was used for comparison. Since the antifungal drug was not available, so it was not possible to compare the activity of the test materials with that of the standard antifungal agent.

Blank discs were used as negative controls, which ensure that the residual solvents and the filter paper were not active themselves.

The crude extracts were dissolved in sufficient amount of the respective solvents so that each 15 μl of solutions contained 400 μg of the test materials and each of the pure compounds was dissolved in sufficient volume of suitable solvents to get a concentration of 200 μg/15 μl. On the other hand, 20 μg/15 μl of each of lectins in Tris-HCl buffer was used for antibacterial screening. The antibacterial and antifungal activities were determined by the Standard Disc-Diffusion Method (Barry, 1980; Bauer *et. al.,* 1966) by measuring the diameter of the inhibitory zones in mm using a transparent scale. The diameters of the zones of inhibition of the samples were then compared with the diameter of the zone of inhibition produced by the standard antibiotic disc used.

6.1.2.5 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations (MICs) of two pure compounds CBC-2 and CBC-3were determined against *Bacillus subtilis* and *Shigella sonnei* as well as *Bacillus subtilis* and *Shigella dysenteriae* respectively by the Serial Dilution Technique (Reiner, 1982) using nutrient broth medium. 1.024 mg of each of the pure compounds were dissolved individually in 1 ml of suitable solvents so that a solution was obtained a concentration of 1.024 mg / ml each. The lowest concentration of the test sample at which there was no visible growth was noted as the MIC of the test sample for the test organism.

6.2 Brine Shrimp Lethality : A rapid general bioassay for bio-active compounds (Mayer *et al*., 1982, Persone, 1980).

Brine shrimp lethality bioassay was carried out using brine shrimp eggs. Shrimp eggs were placed in one side of a small tank divided by a net containing seawater (3.8 % NaCl solution) for hatching. In the other side of the tank a light source was

placed in order to attract the nauplii. Two days were allowed to hatch all the eggs and in this period the nauplii were also sufficiently matured for experiment.

The crude extracts and pure compounds obtained from *Cassia fistula* were dissolved separately in sufficient volume of dimethyl sulfoxide (DMSO) so that each 1μl of solutions contained 10 μg of the test materials. From the stock solutions of the samples, specific volumes were transferred to the different vials containing 10 living shrimps and brine water was added to make the volumes up to 5 ml in each vial to get the final sample concentrations of 5, 10, 20, 40, 80 μg/ml. Three experiments were carried out for the same concentration to get more accurate result. A control experiment was also performed similarly taking 10 living shrimps in 5 ml seawater. After 24 hours incubation, the vials were observed and the number of death in each vial was counted using a magnifying glass and noted. From this data, the mean percentage of mortality of the nauplii was calculated for each concentration.

6.3 Results and Discussion

In vitro Antibacterial Screening

In order to detect the antibacterial activity of a new leading compound for development as potential new drug *in vitro* antibacterial screening is a useful technique. In general, antibacterial screening is undertaken into two phases: a primary qualitative assay to detect the presence or absence of activity and a secondary assay which quantifies the relative potency expressed as minimum inhibitory concentration (MIC) value of any compound.

The different crude extracts, pure compounds and lectins were tested for their antibacterial activities against a number of gram-positive and gram-negative bacteria and the results were furnished in Table-6.2, 6.3, 6.4, 6.5 & 6.6

Table-6.2: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* stem bark and standard antibiotic Kanamycin against bacterial strains.

Name of bacteria	Diameter of the zone of inhibition in mm			
	Petroleum ether extract (400 µg / disc)	Ethyl acetate extract (400µg/disc)	Methanol extract (400µg/disc)	Standard antibiotic Kanamycin (30µg/disc)
Gram-positive				
Bacillus subtilis	15	18	11	20
Bacillus megaterium	10	14	11	14
Streptococcus β-haemolyticus	14	14	14	14
Streptococcus aureus	14	11	-	35
Sarcina lutea	15	15	12	20
Gram-negative				
Shigella sonnei	14	18	10	13
Escherichia coli	11	11	08	30
Klebsiella species	11	17	11	16
Shigella shiga	14	14	08	20
Shigella boydii	23	11	08	35
Shigella flexneriae	10	16	09	15
Shigella dysenteriae	15	14	15	30
Salmonella typhi	12	19	09	19
Pseudomonas aeruginosa	08	12	08	15

- = Inactive against organisms

Table-6.3: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* leaves and standard antibiotic Kanamycin against bacterial strains.

Name of bacteria	Diameter of the zone of inhibition in mm			
	Petroleum ether extract (400 µg / disc)	Ethyl acetate extract (400µg/disc)	Methanol extract (400µg/disc)	Standard antibiotic Kanamycin (30µg/disc)
Gram-positive				
Bacillus subtilis	09	10	-	20
Bacillus megaterium	09	08	12	14
Streptococcus β-haemolyticus	07	12	15	14
Streptococcus aureus	09	06	-	35
Sarcina lutea	12	08	-	20
Gram-negative				
Shigella sonnei	10	07	-	13
Escherichia coli	09	12	-	30
Klebsiella species	09	10	-	16
Shigella shiga	10	09	-	20
Shigella boydii	09	08	-	35
Shigella flexneriae	09	10	-	15
Shigella dysenteriae	14	-	-	30
Salmonella typhi	09	10	09	19
Pseudomonas aeruginosa	07	10	12	15

- = Inactive against organisms

Table-6.4: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* pod and standard antibiotic Kanamycin against bacterial strains.

Name of bacteria	Diameter of the zone of inhibition in mm			
	Petroleum ether extract (400 µg / disc)	Ethyl acetate extract (400µg/disc)	Methanol extract (400µg/disc)	Standard antibiotic Kanamycin (30µg/disc)
Gram-positive				
Bacillus subtilis	12	16	07	20
Bacillus megaterium	16	11	16	14
Streptococcusβ-haemolyticus	11	16	13	14
Streptococcus aureus	11	10	12	35
Sarcina lutea	16	16	10	20
Gram-negative				
Shigella sonnei	16	15	12	13
Escherichia coli	13	10	14	30
Klebsiella species	16	15	11	16
Shigella shiga	13	13	10	20
Shigella boydii	13	13	12	35
Shigella flexneriae	14	13	11	15
Shigella dysenteriae	16	12	09	30
Salmonella typhi	15	14	14	19
Pseudomonas aeruginosa	14	13	12	15

- = Inactive against organisms

Table-6.5: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* seed and standard antibiotic Kanamycin against bacterial strains.

Name of bacteria	Diameter of the zone of inhibition in mm			
	Petroleum ether extract (400 µg / disc)	Ethyl acetate extract (400µg/disc)	Methanol extract (400µg/disc)	Standard antibiotic Kanamycin (30µg/disc)
Gram-positive				
Bacillus subtilis	-	09	08	20
Bacillus megaterium	-	07	09	14
Streptococcus β-haemolyticus	-	07	10	14
Streptococcus aureus	-	-	-	35
Sarcina lutea	-	07	10	20
Gram-negative				
Shigella sonnei	-	07	08	13
Escherichia coli	-	07	12	30
Klebsiella species	-	08	09	16
Shigella shiga	-	07	09	20
Shigella boydii	-	07	11	35
Shigella flexneriae	-	09	07	15
Shigella dysenteriae	-	09	13	30
Salmonella typhi	-	-	-	19
Pseudomonas aeruginosa	-	10	09	15

- = Inactive against organisms

Table-6.6: Zone of inhibition exhibited by the compounds CBC-2 & CBC-3 purified from *Cassia fistula* stem bark as well as lectins CSL-1, CSL-2 & CSL-3 purified from *Cassia fistula* seeds and standard antibiotic Kanamycin against bacterial strains.

Name of bacteria	Diameter of the zone of inhibition in mm					
	CBC-2 (200 µg / disc)	CBC-3 (200µg/ disc)	CSL-1 (20 µg / disc)	CSL-2 (20 µg / disc)	CSL-3 (20 µg / disc)	Standard antibiotic Kanamycin (30µg/disc)
Gram-positive						
Bacillus subtilis	15	17	-	-	07	20
Bacillus megaterium	-	-	-	08	20	14
Streptococcus β-haemolyticus	07	12	08	22	20	14
Streptococcus aureus	12	14	-	07	10	35
Sarcina lutea	-	10	07	07	13	20
Gram-negative						
Shigella sonnei	17	16	-	08	12	13
Escherichia coli	13	15	-	08	08	30
Klebsiella species	08	14	-	07	08	16
Shigella shiga	11	14	-	07	08	20
Shigella boydii	07	09	-	-	17	35
Shigella flexneriae	10	15	-	07	12	15
Shigella dysenteriae	08	18	-	07	15	30
Salmonella typhi	-	10	-	07	10	19
Pseudomonas aeruginosa	-	08	-	-	12	15

- = Inactive against organisms

As shown in Table-6.2, all the three extracts (pet. ether, ethyl acetate and methanol) obtained from *Cassia fistula* stem bark showed mild to strong activity against most of the tested bacteria. The results were compared with those of kanamycin as a standard antibiotic. Of the three extracts, only methanol extract did not show any activity against gram-positive *Streptococcus aureus*. Petroleum ether extract displayed excellent activity against gram-negative *Shigella boydii* whereas ethyl acetate extract showed very strong activity against gram-positive *Bacillus subtilis* and gram-negative *Shigella sonnei*. But the activities, on overall consideration, of methanol extract were not so enough as those of petroleum ether and ethyl acetate extracts.

From the Table-6.3, It is evident that the extracts of *Cassia fistula* leaves were found to be mild active against most of the bacterial strains. Although, of the three extracts, methanol extract is inactive against most of the bacteria tested it showed strong activity against gram-positive *Streptococcusβ-haemolyticus*. On the other hand, ethyl acetate extract displayed activity against all the bacterial strains except *Shigella dysenteriae* and petroleum ether extract was active against all the tested bacteria.

Results depicted in Table-6.4, demonstrate that all the three extracts extracted from *Cassia fistula* pod displayed mild to strong activity against most of the bacteria tested. In this screening work, no extract was found to be inactive against any organism. But the methanol extract exhibited comparatively lower activity against most of the bacteria tested than that of the other two.

As shown in Table-6.5, of the three extracts obtained from *Cassia fistula* seeds, ethyl acetate and methanol extracts displayed mild activity against most of the bacteria tested. But petroleum ether extract was found to be inactive against all the bacterial strains. The organisms such as gram-positive *Streptococcus aureus* and gram-negative *Salmonella typhi* were resistant to all the extracts.

Table-6.6 shows the antibacterial activities of compounds CBC-2 & CBC-3 purified from *Cassia fistula* stem bark as well as lectins CSL-1, CSL-2 & CSL-3 purified from *Cassia fistula* seeds. It reveals that the compound CBC-2 did not show any activity against gram-positive *Bacillus megaterium* and *Sarcina lutea* as well as gram-negative *Salmonella typhi* and *Pseudomonas aeruginosa*. But it showed strong activity against gram-positive *Bacillus subtilis* and gram-negative *Shigella sonnei*. The compound CBC-3 was found active against all the bacterial strains except *Bacillus megaterium* and was strongly active against gram-positive *Bacillus subtilis* and gram-negative *Shigella dysenteriae*. On the other hand, of the three lectins, CSL-3 exhibited mild to excellent activity against most of the bacteria tested. The lectin CSL-2 showed poor activity against most of the bacterial strains and displayed strong activity against only gram-positive *Streptococcusβ-haemolyticus*. But the lectin CSL-1 was found to be inactive against all the bacterial strains except gram-positives *Streptococcusβ-haemolyticus* and *Sarcina lutea*.

In vitro Antifungal Screening

The activities of the crude petroleum ether, ethyl acetate and methanol extracts as well as two pure compounds CBC-2 and CBC-3 isolated from *Cassia fistula* stem bark were measured by the zone of inhibition which indicated the activity against fungi tested. But since suitable antifungal drug was not available so it was not possible to compare the activity of the test samples with that of the standard antifungal agent. The results obtained are cited in Table-6.7, 6.8, 6.9, 6.10 and 6.11.

Table-6.7: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* stem bark and blank disc against fungal strains.

Name of fungi	Diameter of the zone of inhibition in mm			
	Petroleum ether extract (400 µg / disc)	Ethyl acetate extract (400µg/disc)	Methanol extract (400µg/disc)	Blank disc
Aspergillus niger	-	20	-	-
Aspergillus flavus	-	09	10	-
Aspergillus fumigatus	12	10	11	-
Penecillum notatum	-	-	08	-
Hensinela californica	12	14	09	-
Candida albicans	11	13	-	-

- = Inactive against organisms

Table-6.8: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* leaves and blank disc against fungal strains.

Name of fungi	Diameter of the zone of inhibition in mm			
	Petroleum ether extract (400 µg / disc)	Ethyl acetate extract (400µg/disc)	Methanol extract (400µg/disc)	Blank disc
Aspergillus niger	08	07	09	-
Aspergillus flavus	07	10	09	-
Aspergillus fumigatus	-	-	-	-
Penecillum notatum	09	13	09	-
Hensinela californica	-	-	-	-
Candida albicans	11	10	07	-

- = Inactive against organisms

Table-6.9: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* pod and blank disc against fungal strains.

Name of fungi	Diameter of the zone of inhibition in mm			
	Petroleum ether extract (400 µg / disc)	Ethyl acetate extract (400µg/disc)	Methanol extract (400µg/disc)	Blank disc
Aspergillus niger	-	-	-	-
Aspergillus flavus	11	09	10	-
Aspergillus fumigatus	10	12	09	-
Penecillum notatum	09	13	10	-
Hensinela californica	-	10	-	-
Candida albicans	08	12	11	-

- = Inactive against organisms

Table-6.10: Zone of inhibition exhibited by different solvent extracts of *Cassia fistula* seeds and blank disc against fungal strains.

Name of fungi	Diameter of the zone of inhibition in mm			
	Petroleum ether extract (400 µg / disc)	Ethyl acetate extract (400µg/disc)	Methanol extract (400µg/disc)	Blank disc
Aspergillus niger	-	-	10	-
Aspergillus flavus	-	11	-	-
Aspergillus fumigatus	09	07	-	-
Penecillum notatum	-	08	09	-
Hensinela californica	-	07	10	-
Candida albicans	-	-	-	-

- = Inactive against organisms

Table-6.11: Zone of inhibition exhibited by pure compounds-CBC-2 & CBC-3, isolated from *Cassia fistula* stem bark and blank disc against fungal strains.

Name of fungi	Diameter of the zone of inhibition in mm		
	CBC-2 (200μg/disc)	CBC-3 (200μg/disc)	Blank disc
Schizosporum species	-	10	-
Aspergillus flavus	11	07	-
Aspergillus fumigatus	08	10	-
Aspergillus niger	10	-	-
Hensinela californica	12	09	-
Candida albicans	-	10	-

- = Inactive against organisms

As shown in Table-6.7, the crude petroleum ether extract of *Cassia fistula* stem bark was active against *Aspergillus fumigatus, Hensinela californica* and *Candida albicans* whereas methanol extract was inactive against *Aspergillus niger* and *Candida albicans.* Ethyl acetate extract was found to be significantly active against all the fungal strains except *Penecillum notatum.* It exhibited strong activity against *Aspergillus niger.* The results mentioned in Table-6.8 demonstrated that all the three extracts of *Cassia fistula* leaves displayed mild to moderate activity against some of the tested fungi. But they have no activity against *Aspergillus fumigatus* and *Hensinela californica.* From the Table-6.9, it is evident that all the extracts showed mild to moderate activity against most of the fungal strains. The fungus such as *Aspergillus niger* was resistant against all the

crude extracts whereas *Hensinela californica* was resistant to petroleum ether and methanol extract.

Table-6.10 shows that all the extracts, obtained from *Cassia fistula* seeds displayed little activity against some of the fungal strains. Among them, petroleum ether extract was found to be active against only the organism *Aspergillus fumigatus* whereas ethyl acetate extract was inactive against *Aspergillus niger* and *Candida albicans*. Table-6.11 describes the activities of two isolated pure compounds CBC-2 and CBC-3 against fungal strains. Compound CBC-2 did not show activity against *Schizosporum species* and *Candida albicans* and compound CBC-3 was resistant to all the organisms except *Aspergillus niger.*

Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) is the lowest concentration of the test sample, which inhibits clearly visible growth of the test organisms. It was determined by Serial Dilution Assay technique, which quantifies the antimicrobial activities by providing the MIC value of the compounds for specific susceptible test organisms and important consideration in the further development of bio-active compounds. The MIC values were determined against some of pathogenic bacteria for pure compounds CBC-2 and CBC-3, which were found to be potent against these bacteria.

The first sign of inhibition was seen in the test tube containing :

32 µg/ml in the case of gram- positive *Bacillus subtilis* for pure compound CBC-2 isolated from *Cassia fistula* stem bark.

32 µg/ml in the case of gram-negative *Shigella sonnei* for pure compound CBC-2 isolated from *Cassia fistula* stem bark.

64 µg/ml in the case of gram- positive *Bacillus subtilis* for pure compound CBC-3 isolated from *Cassia fistula* stem bark.

32 μg/ml in the case of gram- negative *Shigella dysenteriae* for pure compound CBC-3 isolated from *Cassia fistula* stem bark.

No inhibition was recorded in the test tubes containing test sample lower than the above concentrations. Growth of the organisms was observed in the test tube C_I and contents of the test tubes C_S and C_M were clear. The results thus obtained are furnished in Table-6.12 and 6.13.

Table-6.12: Minimum inhibitory concentrations (MICs) of the pure compound CBC-2 isolated from *Cassia fistula* stem bark against gram-positive *Bacillus subtilis* and gram-negative *Shigella sonnei*.

No. of test tube	Nutrient broth medium added (ml)	Diluted solution of compound (μg/ml)	Inoculum added (μl)	Observation	
				Bacillus subtilis	*Shigella sonnei*
1	1	512	10	NG	NG
2	1	256	10	NG	NG
3	1	128	10	NG	NG
4	1	64	10	NG	NG
5	1	32	10	NG	NG
6	1	16	10	G	G
7	1	8	10	G	G
8	1	4	10	G	G
9	1	2	10	G	G
10	1	1	10	G	G
C_S	1	512	0	NG	NG
C_I	1	0	10	G	G
C_M	1	0	0	NG	NG

G = Growth
NG = No growth
No. of cells = 10^7 cells/ml

C_S = Medium plus extract
C_I = Medium plus inoculum
C_M = Medium only

From the Table-6.12, it was found that the minimum inhibitory concentrations of pure compound CBC-2 against *Bacillus subtilis* and *Shigella sonnei* were the same, i. e., 32 μg/ml.

Table-6.13: Minimum inhibitory concentrations (MICs) of the pure compound CBC-3 isolated from *Cassia fistula* stem bark against gram-positive *Bacillus subtilis* and gram-negative *Shigella dysenteriae*.

No. of test tube	Nutrient broth medium added (ml)	Diluted solution of compound (μg/ml)	Inoculum added (μl)	Observation	
				Bacillus subtilis	*Shigella dysenteriae*
1	1	512	10	NG	NG
2	1	256	10	NG	NG
3	1	128	10	NG	NG
4	1	64	10	NG	NG
5	1	32	10	G	NG
6	1	16	10	G	G
7	1	8	10	G	G
8	1	4	10	G	G
9	1	2	10	G	G
10	1	1	10	G	G
C_S	1	512	0	NG	NG
C_I	1	0	10	G	G
C_M	1	0	0	NG	NG

G = Growth

NG = No growth

No. of cells = 10^7 cells/ml

C_S = Medium plus extract

C_I = Medium plus inoculum

C_M = Medium only

As shown in Table-6.13, the minimum inhibitory concentrations of pure compound CBC-3 against *Bacillus subtilis* and *Shigella dysenteriae* were 64 and 32 μg/ml respectively.

Brine Shrimp Lethality Bioassay

Brine shrimp lethality bioassay is a recent development in the bioassay for bioactive compounds, which indicates cytotoxicity as well as a wide range of pharmacological activities of the compounds. Pharmacology is simply toxicology at lower doses, or toxicology is simply pharmacology at higher doses. Natural product extracts and pure compounds can be tested for their bioactivities by this method. Here, *in vivo* lethality, a simple zoological organism is used as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products.

In this bioassay, the crude extracts and pure compounds obtained from *Cassia fistula* were screened for probable cytotoxic activity. All the extracts and compounds showed positive results indicating that they were biologically active. From this experiment, it is revealed that each of the test samples showed different mortality rates at different concentrations. The mortality rate of brine shrimp was found to be increased with the increase of the concentration of the sample and a plot of log of concentration versus percentage of mortality on the graph paper produced an approximate linear correlation between them. From the graph, the concentration at which 50 % mortality (LC_{50}) of brine shrimp nauplii occurred was obtained by extrapolation. The results are furnished in Table-6.14, 6.15, 6.16 and 6.17.

Table-6.14: Effect of crude extracts of *Cassia fistula* stem bark on brine shrimp lethality bioassay.

Test sample	Conc. (µg/ml)	Log conc. (Log C)	No. of shrimp taken	No. of shrimp death			Avarage No. of death	Mortality (%)	LC_{50} (µg/ml)
				Vial 1	Vial 2	Vial 3			
Nil (Control)	0	0	10	0	0	0	0	0	
Petroleum ether extract	5	0.699	10	3	3	3	3.000	30.00	13.85
	10	1.000	10	4	4	5	4.333	43.33	
	20	1.301	10	6	5	6	5.666	56.66	
	40	1.602	10	7	7	7	7.000	70.00	
	80	1.903	10	8	9	9	8.666	86.66	
ethyl acetate extract	5	0.699	10	3	3	4	3.333	33.33	11.00
	10	1.000	10	4	5	5	4.666	46.66	
	20	1.301	10	6	6	7	6.333	63.33	
	40	1.602	10	8	8	7	7.666	76.66	
	80	1.903	10	10	9	9	9.333	93.33	
Methanol extract	5	0.699	10	3	3	2	2.666	26.66	17.64
	10	1.000	10	4	4	4	4.000	40.00	
	20	1.301	10	6	5	6	5.666	56.66	
	40	1.602	10	6	7	7	6.666	66.66	
	80	1.903	10	8	8	8	8.000	80.00	

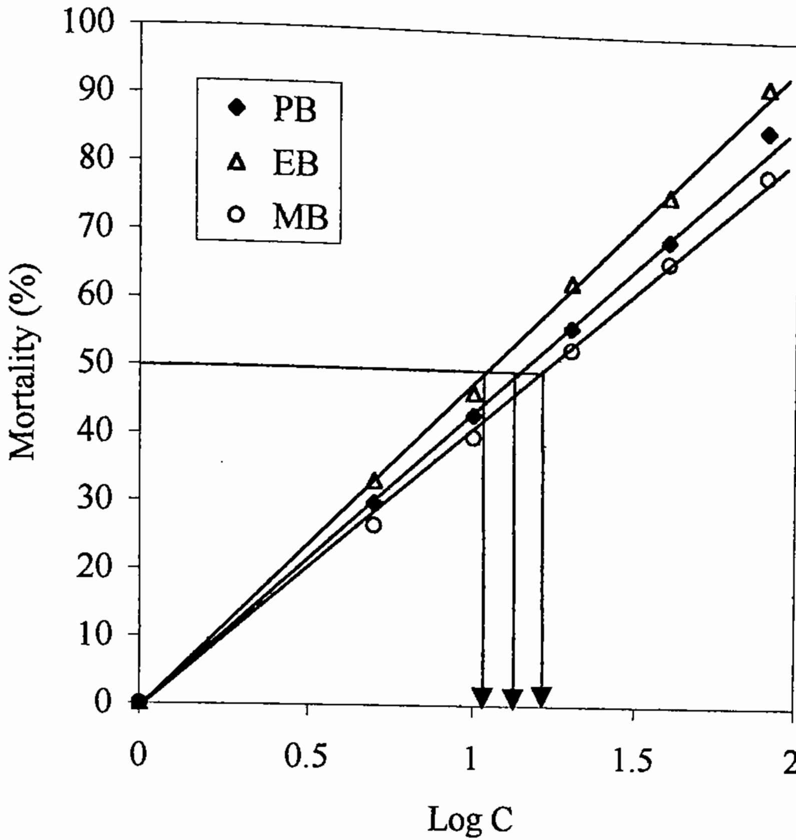


Figure-6.1: Determination of LC_{50} of crude extracts of *Cassia fistula* stem bark

PB-Petroleum ether extract EB-Ethyl acetate extract MB-Methanol extract

It is evident from the Table-6.14 that all the extracts obtained from *Cassia fistula* stem bark affect significantly the mortality rate on brine shrimp. From the graph (Figure-6.1), the LC_{50} as estimated by the extrapolation was found to be 13.85 μg/ml for petroleum ether extract, 11.00 μg/ml for ethyl acetate extract and 17.64 μg/ml for methanol extract.

Table-6.15: Effect of crude extracts of *Cassia fistula* leaves on brine shrimp lethality bioassay.

Test sample	Conc. (µg/ml)	Log conc. (Log C)	No. of shrimp taken	No. of shrimp death			Avarage No. of death	Mortality (%)	LC_{50} (µg/ml)
				Vial 1	Vial 2	Vial 3			
Nil (Control)	0	0	10	0	0	0	0	0	
Petroleum ether extract	5	0.699	10	3	3	3	3.000	30.00	13.96
	10	1.000	10	4	4	5	4.333	43.33	
	20	1.301	10	5	6	5	5.333	53.33	
	40	1.602	10	7	7	7	7.000	70.00	
	80	1.903	10	8	8	9	8.333	83.33	
ethyl acetate extract	5	0.699	10	2	2	3	2.333	23.33	23.26
	10	1.000	10	3	3	4	3.333	33.33	
	20	1.301	10	4	5	5	4.666	46.66	
	40	1.602	10	6	6	6	6.000	60.00	
	80	1.903	10	7	7	7	7.000	70.00	
Methanol Extract	5	0.699	10	2	3	3	2.666	26.66	18.05
	10	1.000	10	4	4	3	3.666	36.66	
	20	1.301	10	5	5	5	5.000	50.00	
	40	1.602	10	7	7	6	6.666	66.66	
	80	1.903	10	8	7	8	7.666	76.66	

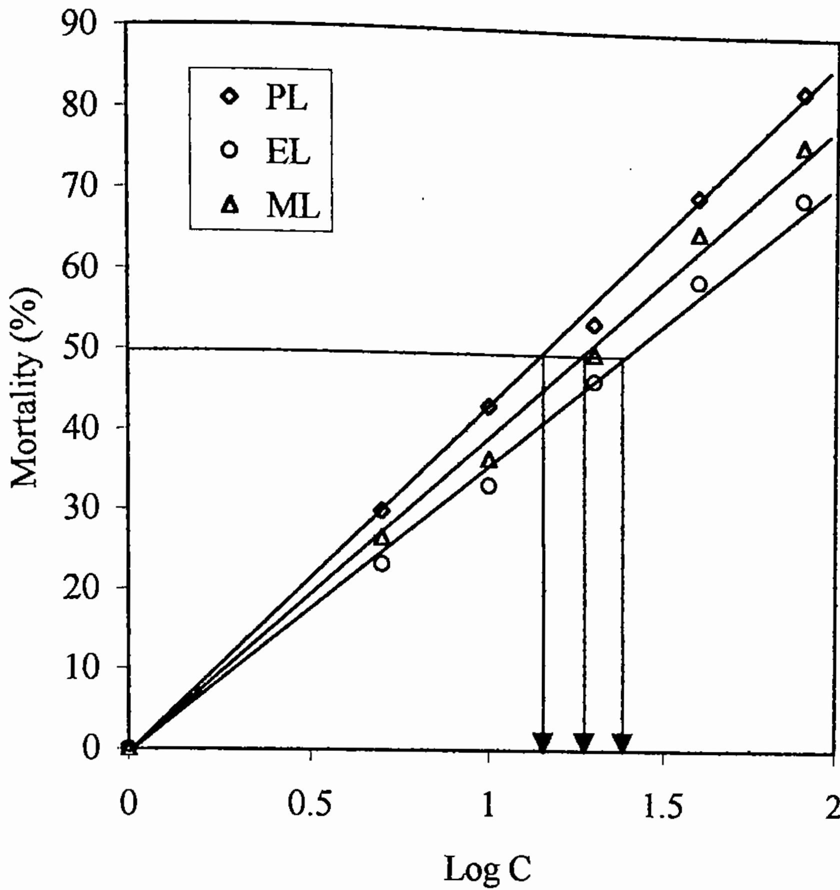


Figure-6.2: Determination of LC_{50} of crude extracts of *Cassia fistula* leaves

PL-Petroleum ether extract EL-Ethyl acetate extract ML-Methanol extract

As shown in Table-6.15, all the extracts of *Cassia fistula* leaves exhibited positive results in brine shrimp lethality bioassay. From the graph (Figure-6.2), the LC_{50} was found to be 13.96 μg/ml for petroleum ether extract, 23.26 μg/ml for ethyl acetate extract and 18.05 μg/ml for methanol extract.

Table-6.16: Effect of crude extracts of *Cassia fistula* pod on brine shrimp lethality bioassay

Test sample	Conc. (µg/ml)	Log conc. (Log C)	No. of shrimp taken	No. of shrimp death			Avarage No of death	Mortality (%)	LC_{50} (µg/ml)
				Vial 1	Vial 2	Vial 3			
Nil (Control)	0	0	10	0	0	0	0	0	
Petroleum ether Extract	5	0.699	10	3	3	3	3.000	30.00	16.10
	10	1.000	10	4	4	4	4.000	40.00	
	20	1.301	10	6	5	5	5.333	53.33	
	40	1.602	10	7	7	6	6.666	66.66	
	80	1.903	10	8	8	8	8.000	80.00	
Ethyl acetate extract	5	0.699	10	3	3	4	3.333	33.33	9.99
	10	1.000	10	5	5	5	5.000	50.00	
	20	1.301	10	6	7	7	6.666	66.66	
	40	1.602	10	8	8	8	8.000	80.00	
	80	1.903	10	9	10	9	9.333	93.33	
Methanol extract	5	0.699	10	3	2	3	2.666	26.66	19.49
	10	1.000	10	4	4	3	3.666	36.66	
	20	1.301	10	5	5	5	5.000	50.00	
	40	1.602	10	6	6	7	6.333	63.33	
	80	1.903	10	7	8	8	7.666	76.66	

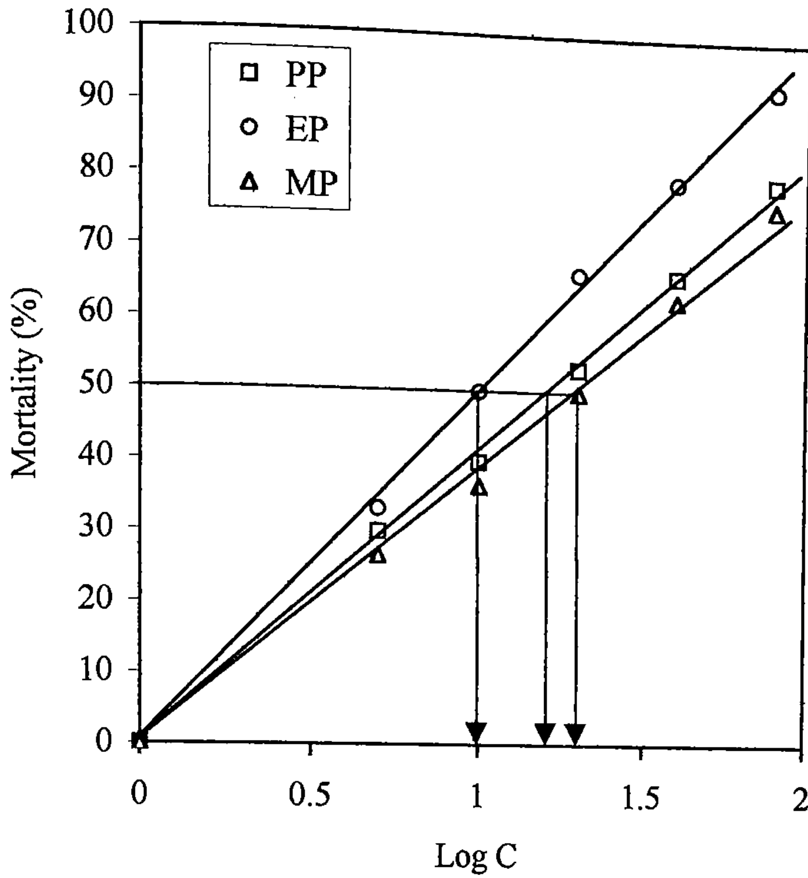


Figure-6.3: Determination of LC_{50} of crude extracts of *Cassia fistula* pod

PP-Petroleum ether extract EP-Ethyl acetate extract MP-Methanol extract

As shown in Table-6.16, all the extracts obtained from *Cassia fistula* pod displayed positive results in brine shrimp lethality bioassay. Among them the ethyl acetate extract was found to be highly toxic (9.99 μg/ml) followed by petroleum ether extract (16.10 μg/ml) and then methanol extract (19.49 μg/ml) (Figure-6.3).

Table-6.17: Effect of Pure compounds isolated from *Cassia fistula* stem bark on brine shrimp lethality bioassay.

Test sample	Conc. (µg/ml)	Log conc. (Log C)	No. of shrimp taken	No. of shrimp death			Avarage No. of death	Mortality (%)	LC_{50} (µg/ml)
				Vial 1	Vial 2	Vial 3			
Nil (Control)	0	0	10	0	0	0	0	0	
CBC-2	5	0.699	10	2	3	3	2.666	26.66	14.01
	10	1.000	10	4	4	5	4.333	43.33	
	20	1.301	10	6	5	6	5.666	56.66	
	40	1.602	10	7	7	7	7.000	70.00	
	80	1.903	10	8	9	8	8.333	83.33	
CBC-3	5	0.699	10	3	3	4	3.333	33.33	10.39
	10	1.000	10	4	5	5	4.666	46.66	
	20	1.301	10	7	6	7	6.666	66.66	
	40	1.602	10	8	8	8	8.000	80.00	
	80	1.903	10	9	9	10	9.333	93.33	

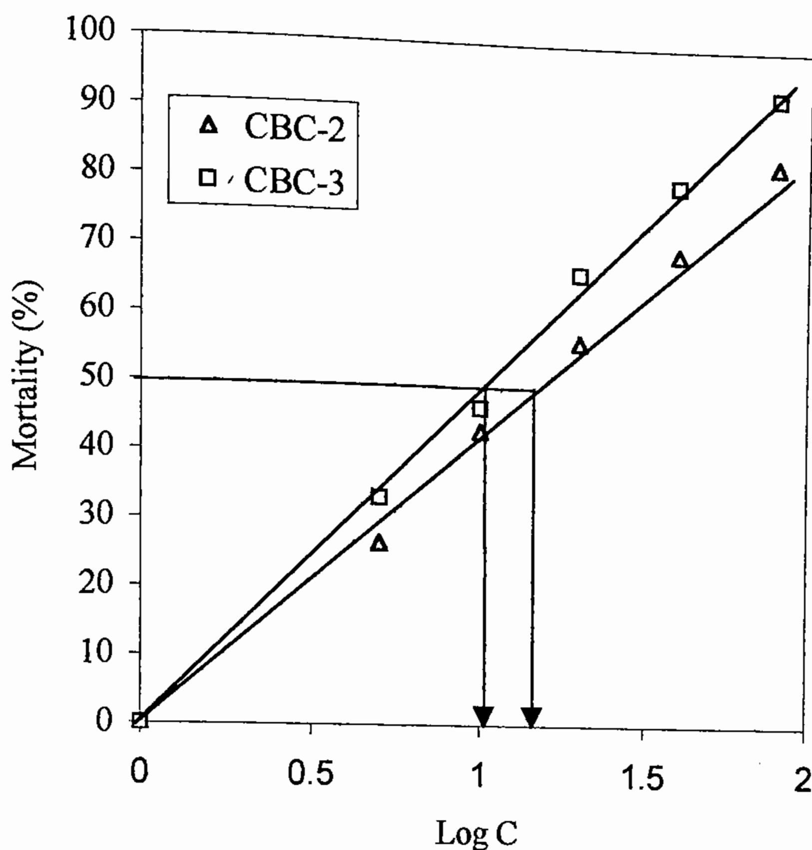


Figure-6.4: Determination of LC_{50} of pure compounds CBC-2 and CBC-3 isolated from *Cassia fistula* stem bark.

As shown in Table-6.17, The LC_{50} values of pure compounds CBC-2 and CBC-3 isolated from *Cassia fistula* stem bark were determined from the graph (Figure-6.4) and found to be 14.01 and 10.39 μg/ml respectively.

Chapter-7

Biochemical Analysis of *Cassia fistula* Seeds and Leaves

Biochemical Analysis of *Cassia fistula* Seeds and Leaves

7.1 Introduction

A large number of populations in our country have been suffering from malnutrition. There are many kinds of vegetable available in our country, which are rich in nutrients. For the ignorance of people, they do not know the nutritive value of different kinds of vegetables. Although numerous toxic materials are found in many pulp of pod and oil seeds, many of them are destroyed by adequate treatment, such as might be employed in common cooking procedure prior to consumption. Cotton seed meal contains a toxic substance called gossypol. By cooking the seeds with at least 14.5 % of moisture at 115 ^{0}C for 90 minutes, the free gossypol is destroyed and the product is then suitable for animal food. Uses of different plant materials as the source of human and animal food have been studied in many countries to combat the nutrients. Barthakur *et. al.* (1995) analyzed the edible fruit tissue of Indian laburnum fruit (*Cassia fistula* L.), a member of the leguminosae family for certain organic compounds and mineral nutrients. The results of their experiments demonstrate that the Indian laburnum fruit may be a source of some important nutrients and energy for humans.

In addition to this, *Cassia fistula* seed is a rich source of protein (Roskoski *et. al.*,1980) which may be considered as a very important economic source of protein. The tree *Cassia fistula* Linn is grown almost in all the districts of Bangladesh. Its curative properties are, in fact, so well known that the *Cassia fistula* tree plays an important role in a village dispensary from the time immemorial.

Till now no detailed studies were done on the biochemical analysis of *Cassia fistula*. Hence the present investigations were carried out with a view to finding out the proximate biochemical compositions of seeds and leaves obtained from locally available plant *Cassia fistula* at the location of Bangladesh typical in subcontinent, under the influence of variegated climates, monsoons, moisture conditions etc.

7.2 Materials and Methods

7.2.1 Materials

The ripe fruits and the matured leaves were collected from the same tree *Cassia fistula* planted at Rajshahi University Campus, Rajshahi. The seeds were separated from the fruits and adhering particles were removed from the seeds. Air-dried seeds and fresh leaves were used for present experimental purpose and the results were depicted as the mean value of three replicates. All the reagents used were of analytical grade unless otherwise specified.

7.2.2 Determination of pH

2 grams of *Cassia fistula* leaves were crushed thoroughly in a mortar with a pestle and homogenized well with 30 ml of distilled water and then filtered through two layers of muslin cloth. The filtrate was then centrifuged for 10 min. at 5000 g and the clear supernatant was collected. The pH of the extracted juice was determined by a pH meter using standard buffer solution.

7.3.3 Determination of Total Titratable Acidity (TTA)

The juice of *Cassia fistula* leaves was extracted by the procedure as described earlier and the total titratable acidity of the extracted juice was determined by Folin's method (Rangannas, 1976).

7.2.4 Estimation of Chlorophyll

The extraction of chlorophyll was carried out with 80 % acetone from the leaves. After filtration, the filtrate was pooled and made upto 100 ml in a volumetric flask with 80 % acetone and the absorbance of this extract was measured at 645 nm and 663 nm for the determination of chlorophyll-a and chlorophyll-b. The chlorophyll contents were calculated employing the following formula using the specific absorption coefficient for chlorophyll-a and chlorophyll-b at 645 nm and 663 nm in 80 % acetone respectively (Mahadevan and Sridhar, 1982).

$$\text{Total chlorophyll (mg/gm)} = \frac{20.2\ A_{645} + 8.02\ A_{663}}{l \times 1000 \times w} \times v$$

$$\text{Chlorophyll-a (mg/gm)} = \frac{12.7\ A_{663} - 2.69\ A_{645}}{l \times 1000 \times w} \times v$$

$$\text{Chlorophyll-b (mg/gm)} = \frac{22.9\ A_{645} - 4.68 A_{663}}{l \times 1000 \times w} \times v$$

Where, A = Optical density in each case

l = Length of light path in the cell

v = Volume of the extract in ml

and w = Weight of the sample in gram.

7.2.5 Estimation of Total Phenol Content

Extraction of phenol from *Cassia fistula* leaves was carried out following the procedure as depicted by Loomis and Shall (1937), using ethyl alcohol. The total phenol content was determined colorimetrically by Folin-Ciocalteu method (Bray and Thorpe, 1954) using catechol as the standard.

7.2.6 Determination of Moisture Content

Moisture contents of seeds and leaves were determined by weight loss of the sample on drying at 105 ^{0}C for 5 hours (ICOMR, 1971).

7.3.7 Determination of Ash Content

Ash contents of seeds and leaves were assessed by the method of AOAC (1980).

7.3.8 Determination of Lipid Content

Lipid contents of seeds and leaves were estimated by the method of Bligh and Dyer (1959) using a solvent mixture of chloroform and ethanol (2:1 v/v).

7.3.9 Determination of Total Protein Content

Total protein contents of seeds and leaves were calculated from total nitrogen by using N × 6.25 after determination of the total nitrogen by micro-Kjeldhal method (Wong, 1923)

7.3.10 Determination of Water Soluble Protein

Water soluble proteins of leaves and seeds were determined by Folin-Lowry method (Lowry *et. al.*, 1951) using Bovine serum albumin as the standard.

7.3.11 Estimation of Crude Fiber

Crude fiber contents of seeds and leaves were estimated by the method of ICOMR (1971)

7.3.12 Estimation of Total Carbohydrate and Energy Value (Rahim *et. al.*, 1999)

The nitrogen free extracts (NFE) were considered as total carbohydrate and was calculated by the following equation: Carbohydrate (gm/100 gm) = 100 – (moisture + protein + lipid +fiber + ash) gm/100 gm. On the other hand, energy value of the samples was estimated and expressed in kilocalories by multiplying the percentage of protein, lipid and carbohydrate by the Atwater-Bryant factors 4, 9 and 4 respectively.

7.3 Results and Discussion

pH and Total Titratable Acidity (TTA)

The pH of the aqueous extract of *Cassia fistula* leaves was found to be slightly acidic i.e. about 6.30. This value was further confirmed from the result of TTA estimation (Table-7.1).

Chlorophyll Content

Chlorophylls are the green pigments universally present in all photosynthetic tissues. Chlorophyll-a and chlorophyll-b occur in higher plants. Disease development affects not only the total chlorophyll content but also alters the ratio between chlorophyll-a and chlorophyll-b. A measurement of green pigments may indirectly denote the severity of disease especially in systemic diseases. Chlorophyll estimation may also be required to relate other biochemical changes in the plant tissue (Mahadevan and Sridhar, 1982). Total Chlorophyll of the *Cassia fistula* leaves was estimated and found to be 186.09 mg % whereas chlorophyll-a and chlorophyll-b were constituted to be 113.97 mg % and 72.12 mg % respectively.

Total Phenol Content

Phenolic compounds enjoy a distribution in the plant kingdom and they are particularly prominent in plants where they are important in determining color and flavor (Buren, 1970). Total phenol content of the *Cassia fistula* leaves was found to be 137 mg %. This value is slightly lower than that of healthy mulberry leaves 145-150 mg % (Tang, Md. Abul Kashem ,2002).

Table-7.1: pH, TTA, Chlorophyll and Total Phenol Contents of the *Cassia fistula* leaves

Parameters	Amount
pH (2 gm/30 ml distilled water)	6.30±0.02
TTA (ml of o.1N NaOH required/100 gm of leaf extract)	17.00±0.06
Total chlorophyll (mg %)	186.09 ± 0.1
Chlorophyll-a (mg %)	113.97± 0.08
Chlorophyll-b (mg %)	72.12± 0.07
Total Phenol (mg %)	137± 0.2

Moisture Content

Moisture plays an important role in the growth activities of plants, herbs etc. Water is indispensable to the absorption and transportation of food to carry on photosynthesis, metabolism of materials and the regulation of temperature. Moisture is also essential for most of the physiological reaction in plant tissue and in its absence, life does not exist (Rangaswami, 1976).

Moisture contents as presented in Table-7.2, of seeds and leaves were estimated to be 7.65 and 65.96 gm % respectively. In the literature (Zaka Shahina *et. al.*, 1988),

it is found that the moisture content of *Cassia fistula* seeds is 7.81 gm % which is very similar to the experimental value. The experimental value for seeds is higher than *Hydnocarpus kurzii* Warb seed 7.00 gm % reported by Faruk Naziba, *et. al.* (1996) but lower than *Xylopia aethiopica* seed (8.43, gm %) depicted by Barminas (1999) and the experimental value for leaves is much lower than the value of 85.12 gm % for *Pterocarpus mildbraedii* Leaves (Akpanyung, 1995).

Ash Content

Ash contents of seeds and leaves were determined and found to be 4.63 and 2.75 gm % respectively (Table-7.2). The FAO (Gohl, 1981) reported that the *Cassia fistula* leaves to contain, on zero moisture basis, 7.8 gm % ash and according to Roskoski *et. al.* (1980) *Cassia fistula* seed contained 4.55 gm % ash which was slightly lower than the value obtained in the seeds under study. In comparison, the percentage composition of ash content estimated from *Cassia fistula* seeds is very close to that for *Teramnus labialis* (L) Spreng seeds 4.62 gm % reported by Viswanathan (1999) and is slightly higher than that for *Hydnocarpus kurzii* Warb seed 4.00 gm % reported by Faruk Naziba *et. al.*, (1996). On the other hand, the percentage composition of ash content found in *Cassia fistula* leaves is lower than that of healthy mulberry leaves 3.05-3.40 gm % as depicted by Tang, Md. Abul Kashem (2002).

Lipid Content

Lipids are essential components of cell membrane, source of metabolic energy for cell maintenance, flight, reproduction and embrayogenesis in insects (Patton, *et. al.*, 1941). Lipid contents of seeds and leaves (shown in Table-7.2) were assayed as 3.85 and 1.88 gm % respectively. The value for lipid content in *Cassia fistula* seeds under experiment is consistent with the value of 4.00 gm % as reported by Zaka Shahina *et. al.* (1988). In comparison, the seeds of *Xylopia aethiopica* (Barminas, 1999) and *Hydnocarpus kurzii* Warb (Faruk Naziba *et. al.*, 1996)

contained 9.58 and 40.00 gm % lipid respectively which are much higher than the percentage of lipid content found in *Cassia fistula* seeds. On the other hand, healthy mulberry leaves contained 1.97-2.18 gm % lipid (Tang, Md. Abul Kashem ,2002) which is slightly higher than that of *Cassia fistula* leaves.

Total Protein and Water Soluble Protein Content

Proteins play crucial roles in virtually all biological processes. The protein constituents of plants and herbs although occurring in low concentrations are of primary importance not only as component of nuclear and cytoplasmic structures but also including as they must be the full complement of enzymes involved in metabolism during growth, development, maturation and the post harvest of the plant (Hansen, 1970).

Total protein contents as depicted in Table-7.2, of seeds and leaves of *Cassia fistula* were constituted to be 22.91 and 5.87 gm % respectively. The present experimental value for seed protein is consistent with the literature value of 24.00 gm % (Roskoski *et. al.*, (1980) and is also nearest to the value of 22.86 gm % for *Teramnus labialis* (L.) Spreng seed (Viswanathan, 1999). But this experimental value for seed protein is lower than the value 27.50 gm % for *Vicia Faba* L. seed (Vetter, 1995) as well as higher than the values 12.45 gm % for *Xylopia aethiopica* seeds (Barminas, 1999) and 19.00 gm % for *Hydnocarpus kurzii* Warb seeds (Faruk Naziba *et. al.*, 1996). The FAO (Gohl, 1981) reported that the leaves of *Cassia fistula* constituted, on zero moisture basis, to be 17.60 gm % of protein which could not be compared with the present experimental value as that value was calculated on zero moisture basis. Moreover, the value obtained in present study for *Cassia fistula* leaves is higher than that of healthy mulberry leaves 4.08-4.62 gm % (Tang, Md. Abul Kashem ,2002). Further 2.91 gm % of total protein found in *Cassia fistula* leaves is water soluble whereas 11.56 gm % of total protein contained in *Cassia fistula* seeds is water soluble.

Findings of the present study clearly indicate that *Cassia fistula* seeds may be used as a good source of protein. The protein content determined by micro-Kjeldahl method showed considerable higher value than that given by the Lowry method. The reason is that Lowry method of protein estimation was applied to a water extract and took into accounts, in this case, the water soluble proteins only. Further, micro-Kjeldahl method takes into account of both the protein and non-protein nitrogen.

Crude Fiber

Fiber is an important component of many complex carbohydrates. It is almost always found only in plants, particularly vegetables, fruits, whole grains, nuts and legumes. As shown in Table-7.2, the crude fiber contents of seeds and leaves were found to be 7.64 and 9.97 gm % respectively. The value for crude fiber content of *Cassia fistula* seeds is higher than the value of 6.68 gm %, reported in the literature (Roskoski *et. al.,* 1980) and this higher value may be due to the climatic and environmental factors, soil texture of the growing areas etc. This value is also higher than that of 4.68-6.92 gm % for *Cassia hirsuta* seeds (Vadivel and Janardhanan, 2000) and lower than the value of 8.66 gm % for *Xylopia aethiopica* seed ((Barminas , 1999). The crude fiber content of *Cassia fistula* leaves as determined, is higher than that of healthy mulberry leaves 6.08-7.04 gm % (Tang, Md. Abul Kashem ,2002). The FAO (Gohl, 1981) reported that the *Cassia fistula* leaves contained, on zero moisture basis, 30.2 gm % fiber.

It may be concluded that the crude fiber contents of the seeds and leaves of *Cassia fistula* were noteworthy and could be considered as good nutritional factor.

Carbohydrate Content and Energy Value

Total carbohydrate contents of seeds and leaves were calculated to be 53.32 and 13.57 gm % respectively (Table-7.2) whereas energy values were estimated to be

339.57 and 94.68 Kcal./100 gm respectively. The present value for carbohydrate content found in *Cassia fistula* seed is slightly higher than that of literature value 50.36 (gm %) as reported by Roskoski *et. al.*, (1980). But this experimantal value is lower than *Xylopia aethiopica* seed 63.65, gm % as reported by Barminas (1999) and also the *Cassia hirsuta* seeds 62.45-70.16, gm % as reported by Vadivel and Janardhanan (2000). On the other hand, the energy value, calculated for *Cassia fistula* seed is lower than those of *Cassia hirsuta* seeds 370.57-390.90, Kcal/100 gm (Vadivel and Janardhanan, 2000) and *Teramnus labialis* (L) Spreng seeds 378.94, kcal/100 gm (Viswanathan, 1999). Barthakur *et. al.* (1995) reported that *Cassia fistula* fruits contained 430.62 Kcal / 100 gm energy value which was higher than the present experimental value. This variation may be possible due to the compositional variation in the soil and climatic condition as well as on the nature of rainfall of the places from where the seed / fruits were collected.

Table-7.2: Major proximate nutritional compositions of seeds and leaves of *Cassia fistula.*

Parameters	Portions of *Cassia fistula* taken for analysis	
	Leaves	Seeds
Moisture (gm %)	65.96 ± 0.08	7.65 ± 0.07
Ash (gm %)	2.75 ± 0.03	4.63 ± 0.06
Lipid (gm %)	1.88 ± 0.03	3.85 ± 0.03
Total protein (N x 6.25) (gm %)	5.87 ± 0.07	22.91 ± 0.09
Water soluble protein (gm %)	2.91 ± 0.05	11.56 ± 0.06
Crude fiber (gm %)	9.97 ± 0.03	7.64 ± 0.05
Total carbohydrate (gm %)	13.57 ± 0.20	53.32 ± 0.30
Energy value (Kcal./100 gm)	94.68 ± 0.20	339.57 ± 0.10

Chapter-8

Conclusion & References

Conclusion

Because of many important biological properties, lectins provide a new and very useful tool for the investigation of specific binding sites on protein molecules and serve as models for the study of antigen-antibody reaction. However, the most important of all is their growing uses in investigating the architecture of cell surfaces and in elucidating the change consequent upon cell malignancy as well as in isolating specific membrane constituents.

Despite the fact that lectins possess a number of interesting properties, only a few have been investigated extensively. From the above stand point, in the present study, three galactose specific lectins CSL-1, CSL-2 and CSL-3 were purified first time, so far as I know, from *Cassia fistula* seeds and were also characterized. It has been known that galactose specific lectins such as ricin, abrin and related toxins are ribosome-inactivating proteins (RIPs). These three newly purified galactose specific lectins may also be tested in future whether they have RIP properties.

Finally, experiments were performed to obtain information on the native structure of the lectins CSL-1, CSL-2 and CSL-3. All the three lectins are very much sensitive to pH values and temperatures. The lectins displayed maximum activities in slightly basic pH values and at the temperature upto 40 ^{0}C, and the activities then decreased sequentially with increasing pH values in the acidic as well as basic ranges and above the temperature 40 ^{0}C which might be helpful to use these lectins for experimental purpose in future under appropriate condition. The other biological activities such as effect of acetic acid, urea and guanidine-HCl were also determined in the present study. These results might be useful to the

Glycobiologist to compare the data on these galactose specific lectins from *Cassia fistula* with those purified from other sources.

From the investigation on the *Cassia fistula* seed oil collected from three different districts (Rajshahi, Dhaka and Rangpur) in Bangladesh, the findings are as follows: the oil can be used as leather dressing and in the manufacture of soap, varnish and paints. But the oil is not tested whether it is edible. Results of fatty acid composition reveal that *Cassia fistula* seed oil is a rich source of linoleic acid, an essential fatty acid, which is important for its manifold uses and can be separated easily by using the multi-stage counter current distribution technique involving urea adducts formation or other methods. If the government takes an initiation to make the oil suitable for consunption, specially for edible purpose, we may get relief from the oil crisis. The present investigations also demonstrate that the characteristics of the oil vary to some extent but not significantly with the locations from where seeds were collected. This variation found in characteristics may be due to the compositional variation in the soil and climatic condition as well as on the nature of rainfall of the places from where seeds were collected.

The powdered materials of stem bark, leaves, pods and seeds of *Cassia fistula* were subjected to extract separately with petroleum ether, ethyl acetate and methanol. Three compounds were isolated from crude ethyl acetate extract of *Cassia fistula* stem bark and were finally identified as β-Sitosterol (CBC-1), Bet-20 (29)-en-3-ol-28-oic acid (CBC-2) and lupeol (CBC-3) on the basis of physico-chemical and spectral evidences. All the crude extracts and pure compounds CBC-2 and CBC-3 were tested for antibacterial and antifungal activities against some pathogenic bacteria and fungi. The MICs of the compounds CBC-2 and CBC-3 were determined against some of the bacterial strains. Three lectins CSL-1, CSL-2 & CSL-3 were also screened for only antibacterial activities against bacterial strains. Though these isolated compounds are known, the present study reports for the first time the antibacterial, antifungal

activity and cytotoxicity of compounds, CBC-2 and CBC-3, isolated from *Cassia fistula* stem bark.

From the antibacterial studies, it is evident that all the extracts (except methanol extract of leaves) of stem bark, leaves and pods exhibited moderate to strong activities against most of the bacteria tested. The extracts of seeds were found to be mild active against most of the bacteria tested whereas its petroleum ether extract was inactive against all the bacterial strains. Of the three lectins, CSL-3 showed strong activity against most of the bacteria tested. But the antifungal activities exhibited by all the crude extracts and compounds CBC-2 & CBC-3 were not significant. It may, therefore, be concluded that the crude extracts as well as isolated pure compounds CBC-2 & CBC-3 may be used enough as drug to treat the disease caused by those bacteria, which are sensitive to the above mentioned samples. But before use in human being isolation of pure compound (in case of only crude extracts), toxicological study and clinical trial in animal model should be carried out thereafter. However, further and specific studies are needed to better evaluate the potential effectiveness of the two isolated compounds CBC-2 & CBC-3 from the *Cassia fistula* stem bark as an antimicrobial agent.

In brine shrimp lethality bioassay, the crude petroleum ether, ethyl acetate and methanol extracts obtained from stem bark, leaves and pods, two pure compounds CBC-2 & CBC-3 as well as three lectins CSL-1, CSL-2 & CSL-3 were screened for probable cytotoxic activity. All the test samples showed different mortality rate at different concentrations and found to be increased with increasing concentration of the sample. It is evident that the pure compounds and pure lectins are comparatively more biologically active than most of the crude extracts. An evaluation of cytotoxicity is also an important investigation for possible clinical use i. e., indicative of wide range of pharmaceutical activities of the drugs.

Biochemical analysis of *Cassia fistula* seeds and leaves was extensively carried out and the data clearly indicated that the seed might be used as a good source of protein since it contains considerable large amount of protein (22.91 %). Crude fiber contents of seeds and leaves of *Cassia fistula* were noteworthy and could also be considered as good nutritional factor.

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