PHARMACOLOGICAL SCREENING OF *SONNERATIA CASEOLARIS* – A MANGROVE PLANT FROM BAGERHAT REGION, THE SUNDARBANS, BANGLADESH.

A Thesis By

Md. Nazmul Hasan
Examination Roll No: MS-090718
Session: 2008-2009

Submitted To

Biotechnology and Genetic Engineering Discipline, Life Science School, Khulna University, Khulna-9208, Bangladesh.
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BIOTECHNOLOGY AND GENETIC ENGINEERING DISCIPLINE
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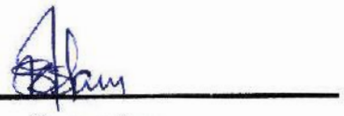
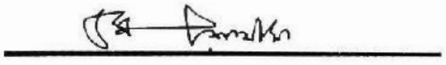
Approved as to the style and content by



Supervisor

Khandker Khaldun Islam

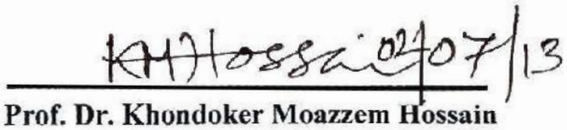
Assistant Professor
Biotechnology and Genetic Engineering
Discipline
Khulna University
Khulna-9208



Co- supervisor

Md. Shamim Akhter

Assistant Professor
Biotechnology and Genetic Engineering
Discipline
Khulna University, Khulna-9208

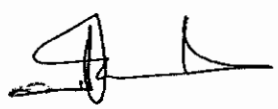


Prof. Dr. Khondoker Moazzem Hossain
Chairman
Examination Committee
Biotechnology and Genetic Engineering Discipline
Khulna University
Khulna-9208

September 2012

DECLARATION

This thesis report is the original research work of the author, except as otherwise stared. The material presented has not submitted either in whole or in part for a degree for this or any other university. The findings of the research has not been/ will not be published anywhere without the concurrence of the concerned supervisor and co-supervisor.



Md. Nazmul Hasan
Student No: MS-090718
September 2012

This thesis is dedicated to my parents

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The Author

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Pharmacological Screening of *Sonneratia caseolaris* - A Mangrove Plant from Bagerhat Region, the Sundarbans, Bangladesh.

Abstract

The present study was designed to investigate the hypoglecemic, analgesic and cytotoxic activities for the methanolic (85%) extracts of fruits, leaves and barks obtained from *Sonneratia caseolaris*. Hypoglycemic tests were done on –Swiss albino mice which were administered with the extracts of *S. caseolaris* at the dose of 50, 100, 200 and 400 mg/kg body weight, respectively in different experimental groups. The mice were sacrificed followed by oral glucose treatment and their sera were collected for glucose level analysis. The results showed that the extracts of *S. caseolaris* were able to decrease blood glucose levels. Especially, leaf extracts showed significant activity in comparison to fruit and bark extracts. In the acetic acid-induced writhing test, the leaf extracts at the dose of 400 mg/kg showed 52.4 % inhibition of writhing, which was significant in comparison to 0.1% acetic acid induction. On the other hand, fruits and barks extracts of *S.caseolaris* did not show response in this regard. The cytotoxic activities of crude extracts (LC_{50}) was determined using brine shrimp lethality bioassay was found to be 0.21±0.19µg/ml. The concentration at which 90% mortality (LC_{90}) of brine shrimp nauplii occurred was obtained by extrapolation & the value was 160µg/ml. The crude leaf extract showed strong lethality against the brine shrimp nauplii. These results are in good agreement with the usage of this plant in antitumor, antimicrobial or pesticidal activities in ethnomedicine.

1.0 Introduction

1.0 INTRODUCTION

The diversity of medicinal plants is growing around the world includes more than a thousand species (Ghani, 1998). More than 500 of these medicinal plants have been reported in Bangladesh. The number of the indigenous medicinal plants continues to grow with discovery and introduction of newer plants everyday (Ghani, 2003). These indigenous medicinal plants have long been extensively used in preparation of unani, ayurvedic and herbal medicines in the country. However, the proper scientific evaluation of the species limits their uses in therapeutic applications.

Mangrove plants have been used as sources of food, a variety of traditional products and artifacts. They have also been used in folk medicine. However, their biological activities and chemical constituents have not yet been studied in detail. *Sonneratia caseolaris* (L.) Engl. is a mangrove plant and belongs to the family Sonneratiaceae (WanJusoh and Hashim, 2009). It is found in the Sundarbans mangrove forest in Bangladesh and is also native of South and South-East Asia. The plant is also reported in Malay Peninsula, Timor, New Guinea, Solomon Islands, and Indonesia and northern Australia (Little, 1983). In English, it is designated asCrabapple while locally it is known as Choilani or Choila.

S. caseolaris has been reported to be used in traditional medicinal systems in several countries. It is used as folk remedy for sprains, swellings, and helminthiasis (Duke and Wain, 1981). In Myammar, the fruit is used for poultices. In Indochina, poultices made of leaves and salt are applied to cuts and bruises. In Malay, old fruit walls are used for helminthiasis, half-ripe fruit for coughs, and pounded leaves are used for hematuria and small pox (Perry, 1980). In Bangladesh, the plant extract are used as astringent and antiseptic agents in sprains and swellings, and in arresting hemorrhage (Ghani, 2003). Young fruits taste sour and are used as flavoring agents. Mature fruits have a cheese-like taste and are eaten raw or cooked. Leaves are sometimes eaten raw. Fermented fruit juice is said to be useful in arresting hemorrhage. The wall of an old fruit is given as a vermifuge. The juice of half-ripe fruit is used to treat coughs. The juice of the flowers enters into a compound for treating blood in the urine. Pneumatophores are used as corks and floats. The crude ethanol extract of leaves of *S. caseolaris* L. (Sonneratiaceae) have

been reported for its antinociceptive and antidiarrhoeal activities. Reported to be hemostat, crabapple mangrove is a folk remedy for sprains, swellings, and worms.

In the effort for identifying anti-diabetic properties from different mangrove flora, it was noticed that methanolic extract of the fresh fruits of *S. caseolaris* possessed intestinal α-glucosidase inhibitory property (Mangala Gowri et al., 2007). In folk medicine, fruit is used as remedy to stop bleeding and in the treatment of piles and cough, removal of intestinal worms and as sprain poultices (Bandaranayake, 2002). It has also been reported to be toxic against mosquito larvae (Devi et al., 1997) and possess hepatoprotective activity (Charoenteeraboon et al., 2007). Fatty acids, sterols, hydrocarbons (Hogg & Gillan, 1984), flavonoid, luteolin and its glycosides (Sadhu et al., 2006) was found in its leaves but chemical and biochemichal properties of the fruits yet to be reported. In origin, *S. caseolaris,* a mangrove plant with potent antioxidant and cytotoxic activity is traditionally used as astringent and antiseptic, sprain poultices, in treating piles (Jayanta et al., 2011).

Brine shrimp lethality bioassay is a recent development in the assay procedure for the bioactive compounds and natural product extracts, which indicates general toxicity as well as a wide range of pharmacological activities e.g. indicative of probable anticancer, antiviral, pesticidal activity (Anderson et al, 1988). Bioactive compounds are usually toxic in high doses. Pharmacology is simply toxicology at a lower dose or toxicology is simply pharmacology at a higher dose. Thus, in-vivo lethality of a simple zoological organism (brine shrimp "nauplii") can be used as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products (Hui et al, 1990). There is a positive correlation between brine shrimp cytotoxicity and doses of plant extracts under investigation.

However, the biological parameters of *S. caseolaris* are yet to be identified. Therefore, the present study was undertaken to identify the pharmacological parameters of extracts from different parts of *S. caseolaris* because there is a possibility that it may contains substances with potential therapeutic uses and these could serve a basis of precursors for synthesis of useful drugs. In addition to these objectives, considering the ethnomedicinal and traditional uses of this plant, it was evaluated for the effect of oral administration of

the methanolic extracts from fruit, leaf and bark on the level of serum glucose in mice. Therefore, the objectives of this study include the followings:

1) Determination of hypoglycemic activity of methanolic extracts of *S. caseolaris*.

2) Determination of analgesic effect of the extracts obtained from this plant.

3) Investigation of the preliminary cytotoxic effects of the leaf extracts of *S. caseolaris*.

3.0 Review of Literature

2.1.2 Botanic description *S. Caseolaris*: Evergreen tree 5–15(–20) m high without buttresses or stilt roots, with rather open spreading crown, glabrous throughout. Pneumatophores 50–90 cm high, to 7 cm in diameter. Bark gray, coarsely flaky. Leaves opposite, without stipules, nearly sessile, elliptical, oblong or ovate, 5–13 cm long, 2–5 cm wide, with broad or tapering base and blunt or rounded tip, entire, with 8–12 widely spreading fine side veins on each side, leathery. Flowers 1–3, at end of drooping twigs malodorous and nocturnal. Hypanthium's with 6–8 calyx lobes; petals 6–8, 2–3.5 cm long, 1.5–3.5 mm wide, dark or blood-red, stamens numerous, with threadlike filaments 2.5–3.5 cm long, pistil with 16–21-celled ovary with many ovules; style long, stout.

Figure 2.2 leaves of *Sonneratia caseolaris*

2.1.3 Habitat of the Experimented Plant: Frequent on tidal mud flats in mangroves, along deep muddy river-banks and from the limit of brackish water down to the lower end of Nypa-zone at river mouths. Its characteristic habitat consists of river banks and tidal areas with mud banks, often in upstream estuarine positions of rivers subjected to large volumes of freshwater run-off or with slow-moving brackish or fresh water and as far inland as the salt water floods.

2.1.4 Ecology: Estimated to range from Tropical Moist to Rain through Subtropical Moist to Rain Forest Life Zones, crabapple mangrove is estimated to tolerate annual precipitation of 10 to 80 dm, annual temperature of 20 to 27°C, and pH of 6.0 to 6.5. Usually on the less salty parts of mangrove forests on a deep muddy soil, never on coral banks, often along tidal creeks with slow moving water, ascending these as far as the flood mounts .

2.1.5 Cultivation: According to the Nasu (2005), planting is usually not needed because natural regeneration is so successful. In Avicennia and Rhizophora, direct seeding results in 90% survival.

2.1.6 Harvesting: Harvested as needed from wild stand. Trees recover rapidly after branches are lopped off for fuel. Since this mangrove can regrow rapidly from buds beneath the bark along the trunk and branches, it is said to suffer little from removal of much of the branch wood.

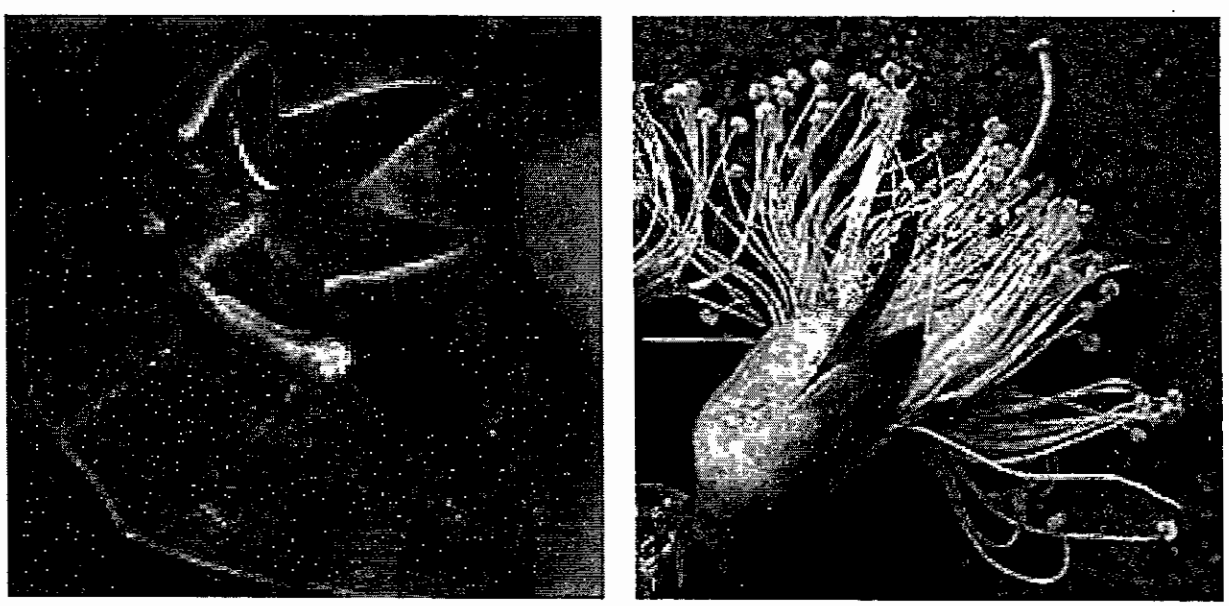

Figure 2.3: Flowers of *Sonneratia caseolaris*

2.1.7 Yields and Economics: Cannell (1982) cites data on a mangrove forest dominated by Rhizophora, Ceriops and Sonneratia, averaging 11 m tall, with an LAI (leaf area Index) of 3.7–4.2. The stem wood and bark on a DM basis weighed 74.4 MT/ha, the prop roots 61.2 MT/ha, the branches 15.8, the foliage 7.4, the fruits 0.3, for a total standing

aerial biomass of 157 MT/ha. The CAI (current annual increment) of stem wood, bark, and branches was 20 MT/ha/yr, foliage 6.7, fruits 0.3. These data, taken from a mangrove on Phuket Island, Thailand, regenerated following clear felling, suggest annual productivity may attain 20 MT/ha/yr in Asian mangroves.

2.1.8 Chemical constituents: Fruits yield 11% pectin (ZMB). Wood yields 52.7% brown pulp (8.5% lignin, 17.6% pentosan). Emodin and chrysophanic acid may be the coloring matter in the crude drug (Perry, 1980). Bark from Africa assayed at 17.1% tannin, of the pyrogallol class. Indian stem bark assayed 9–17%, twig bark 11-12%. Wood yields two coloring principles, archin ($C_{15}H_{10}O_5$) and archinin ($C_{15}H_{14}O_{12}$) (C.S.I.R., 1948–1976).

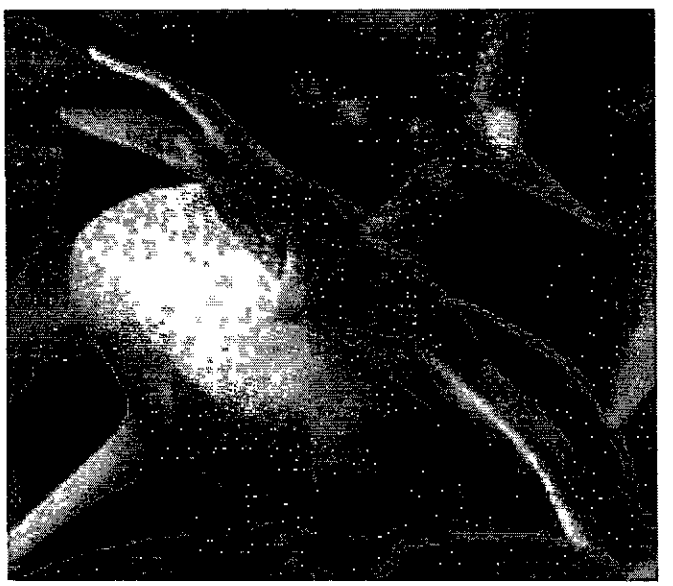

Figure 2.4: Fruit of *Sonneratia caseolaris*

2.1.9 Traditional Use of *S. caseolaris*: Wood used for construction, poles, posts, boat building, and firewood. Young berries are edible and used as a fruit vegetable, pectin can be extracted from the fruits. Young fruits are sour tasting and used as flavoring. Mature fruits have a cheese-like taste and are eaten raw or cooked. They also have medicinal properties. Leaves can be eaten raw.

2.1.10 Pharmacological activity of *S. caseolaris*: Fermented fruit juice is said to be useful in arresting haemorrhage. The wall of an old fruit is given as a vermifuge. The juice of half-ripe fruit is used to treat coughs. The juice of the flowers enters into a compound for treating blood in the urine. Pneumatophores were used as corks and floats. The crude ethanol extract of leaves of *Sonneratia caseolaris* Linn. (Sonneratiaceae) was screened for its antinociceptive and antidiarrhoeal activities. Reported to be hemostat, crabapple mangrove is a folk remedy for sprains, swellings, and worms. Burmese use the fruits for poultices, Indochinese poultice crushed leaves with salt onto cuts and bruises. Malayans use old fruit walls for worms, half-ripe fruits for coughs, and pounded leaves for hematuria and smallpox.

Rasheda Ahmed et al., (2010) reported that Administration of *Sonneratia caseolaris* dried leaf powder to diet of Wistar rats at 0.01, 0.033 and 0.1% (w/w diet) caused a significant dose-dependent decrease in serum glucose levels. At the highest dose administered, there was a more than 20% fall in serum glucose when compared to controls. Administration of leaf powder further caused a dose-dependent significant decreases in serum triglyceride levels and significant decreases in serum total cholesterol and serum low density lipoprotein cholesterol levels. Significant increases in serum high density lipoprotein (HDL)-cholesterol levels were also observed with administration of leaf powder in diets. The triglyceride to HDL-cholesterol ratio fell from 17.4:1 in controls to 7.6:1 at the highest dose of the leaf powder administered. The overall effects suggest that regular intake of leaf powder in diet can have beneficial effects for diabetic and coronary disease patients.

Ashok Kumar Tiwari1 et al, 2008 Showed Fruits of *Sonneratia caseolaris* Linn. have many therapeutic applications in folklore medicine. However, chemical examination and biological activity of its fruit are less studied. During the work on identification of antidiabetic principles from Indian mangrove flora, we noticed moderate intestinal α-glucosidase inhibitory activity of the methanolic extract of its fruits. Three compounds namely oleanolic acid, β-sistosterol-β-D-glucopyranoside and luteolin were isolated and identified from the bioactive methanolic extract. In vitro pre-incubation of crude rat intestinal α-glucosidase with oleanolic acid showed potent α-glucosidase inhibitory (IC50

= 15 μM) activity, however, its inhibitory potential decreased drastically when oleanolic acid was pre-incubated with substrate. In in vivo studies also, pretreatment of rats with oleanolic acid displayed significant ($p<0.05$) antihyperglycemic activity in starch tolerance test however, administration of starch fortified with oleanolic acid to the rats could not exhibit antihyperglycemic activity. This is the first report identifying oleanolic acid in *S. caseolaris* fruits in substantial yield and assigning its intestinal α-glucosidase inhibitory and antihyperglycemic activities.

Sherine George et al., 2010 stated All of these results of bark powder coupled with the effect of chloform extract (bark) reveal that the bark of *Alstonia scholaris* possesses different significant pharmacological activities (Hypoglycemic, hyperlipidemic, anxiolytic & analgesic) at different doses. Shazid M. D. Sharker and Israt Jahan Shahid, 2010 reported Ethanol extracts of the barks of *P. longifolia*, leaves of *E. serratus* and seeds of *T. ammi* showed significant antibacterial activity against some pathogenic bacteria and moderate to mild lethality to the brine shrimps tested. This study provides some scientific bases for the use of this plant as a remedy for stomach, skin and bacterial infections in folkloric medicine whose causative agents are some of the pathogens studies.

Jiny Varghese K et al, 2010 stated that Mangroves are trees and shrubs that grow in tropical and subtropical tidelands throughout the world. Mangroves are able to thrive salt water inundation because of specialized rooting structures. Many of the mangrove species are a rich source of various important phyto constituents and they are useful in microbial infections. Various parts of mangroves are reported to possess medicinal properties. *Sonneratia caseolaris* (family Sonneratiaceae) is a true mangrove plant which is reported to be antioxidant and cytotoxic. It is traditionally used as an astringent and antiseptic, sprain poultices, in treating piles and arresting haemorrhage. In the present investigation, an attempt was made to study the Pharmacognostical and phytochemical features of the mangrove species. Pharmacognostic studies of leaf specimen and leaf powder which include morphological and microscopic characters were carried out. The successive extraction of the plant material was done with different solvents and various extracts were subjected for its preliminary phytochemical screening to find out the bioactive

compounds present in these species. Phytochemical analysis showed the presence of various phytochemicals of therapeutic significance.

2.0 Materials and Methods

3.0 MATERIALS AND METHODS

The study was carried out at Pharmaceutical Biotechnology Laboratory, Department of Genetic Engineering and, Biotechnology, Jessore Science and Technology University (JSTU), Jessore.

3.1 Materials

3.1.1 Animals

A total number of 288 young Swiss-albino female and male mice were used for hypoglycemic and analgesic tests with the extracts of fruit, leaf and bark of *S. caseolaris.* These mice were collected from Animal Resource Branch, ICDDR'B, Mohakhali, Dhaka. They were 4-5 weeks old weighing 20-30 gm on an average. The animals were provided standard laboratory food and tap water and this was maintained at natural day night cycles. The experiments were conducted in an isolated place and noiseless condition was ensured. The mice were housed in standard size metallic cages (8 mice /cage) in properly ventilated room. For hypoglycemic experiments, 144 mice were divided them into 3 groups (48 mice/group) to be treated with fruits, leaves and barks extracts. Each group included 8 mice in which 8 mice in control, 8 mice to be treated with were standard Glibenclamide and 8 each for 4 different doses against body weight (50 mg/kg, 100 mg/kg, 200 mg/kg and 400 mg/kg) respectively.

3.1.2 Materials for extraction

The extractionof *S. caseolaris* fruits, leaves and barks was carried out with methanol. The materials required for this extraction was distilled water, beakers, hot water bath, oven, weighing balance, glass rods, tissue paper, spatula, titrating pipettes, foil papers, vials, Soxhlet apparatus and thimble.

3.1.3 Chemicals

The chemicals used for the spectrometric analysis in determining the serum glucose level in response to oral administration of different plant extracts included reagent of glucose (GOD-PAP Cronolab AG; Swizerland). This reagent was supplied with buffer (Cat no: 101- 0014, GOD-PAP reagents; Lot no: 12679), glucose standard, alcohol, distilled

water, acetone, detergent, ethanol. Other chemicals included sea salt (from fish store), corn oil, di- methyl sulfoxide (DMSO), acetic acid, diclofenac sodium, Castor oil' Loperamid, distilled water, artemia sauna leach (brine shrimp eggs from store).

3.2 Appliances Equipments and Apparatuses

For the proposed study, the equipments and apparatuses used were UV-Spectrophotometer (Beckman-Coultertm; Germany), electronic microbalance (Adventuretm ohans; Ohans Corporation; USA), micropipettes (Gilson Pipette man; France), Vortex Mixture (VM-2000; Digi system; Tiwan), Centrifuge Machine (M# 800; Xiangshui Medical apparatus), Oven (Binder; Germany), refrigerator, glass corvettes and pipettes, test tubes, centrifuge tubes, beakers, glass roads, rope, cloths, dissecting box, safety rubber gloves and disposable gloves, micropipettes (5-200 µl), Eppendroff tubes (1.5 ml) and yellow tips (200 µl), ice box with ice, tubes' holder, funnel; aluminum foil paper and marker, Pipettes (5 ml, 10 ml), small tank with perforated dividing dam for growing shrimps, cover and lamp to attract shrimp etc.

3.3 Procedure for *S. caseolaris* extraction with methanol

Green mature fruits, leaves and bark of *S. caseolaris* were collected from Bagerhat region, the Sundarban's. The collected samples were separated from undesirable materials or plants or plant parts. Then drying of the fruits, leaves and bark was carried out for 15 days under shaded sunlight to prevent the decomposition of the active constituents and any their photochemical degradation. The samples were ground into a coarse powder with the help of a suitable grinder. The powder was then stored in an airtight container and kept in a cool, dark and dry place until analysis was conducted. A porous cellulose thimble was filled with 45gm dried and shredded sample of *S. caseolaris*. The thimble was then placed in an extraction chamber, which was suspended above a flask containing 400 ml methanol solvent and below a condenser was placed. The flask was heated at 45^0C and the solvent evaporated and moved up into the condenser where it was converted inside the extraction chamber containing the sample. The extraction chamber was designed in a manner so that when the solvent surrounding the sample exceeds a certain level, it would overflow and trickle back down into the boiling flask. This created a siphon and the process was repeated 7 times over 6 hours.

The thimble was subsequently filled with 32 gm and then 30gm *S.caseolaris* powder and the whole procedure was repeated. At the end of the extraction process, the flask containing the solvent and lipid was removed from the thimble after squeezing to leave the soaked thimble. The extract residues were dried on tray and covered by aluminum foil paper and the filtered solution (extract) was dried by hot water bath for 3 days at 40^0C. After 3 days a sticky oily precipitate was found in the beaker and the extracts were collected by spatula in the designated glass vials. The amount obtained for different extracts were fruit 9gm, leaves 7gm, and bark 12gm, respectively. The extracted residues were kept in plastic jars and the extract containing vials were kept in the refrigerator at temperature of 7-10^0C.

3.4 Determination of hypoglycemic effects of methanolic extracts of *S. caseolaris* in mice

As mentioned earlier, 48 mice were used for this experiment and they were divided in to 6 groups. First group which was control group contained 8 mice. The second group with 8 mice was treated with standard (Glibenclamide 10mg/kg body weight). The remaining groups were treated with different doses of plant extracts depending on the body weight in which group-3 of 8 mice was administered with 50mg/kg body weight while group-4, 5 and 6, each with 8 mice received the doses of 100mg/kg body weight and 200mg/kg body weight 400mg/kg body weight, respectively. The dose of glucose and Glibenclamide administered in oral glucose test was 2g/kg and 10mg/kg body weight. Before the day of gavaging and serum glucose analysis, the mice were fasted overnight and on the day of gavaging, the methanolic extracts of each sample were weighed in 0.4 gm and then suspended in corn oil. The net volume was prepared as 3ml and the mixture was shaken till the extract was suspended thoroughly. Then 10gm glucose was dissolved in distilled water and net volume was made 10ml.Simultaneously, 5mg/ml Glibenclamide was dissolved in distilled water and net volume was prepared 1ml. The extract or Glibenclamide was administered by gavaging and the mice were kept for 60 minutes before any treatment was carried out. Then glucose solution was administered by gavaging and again the mice were kept for 60 minutes without any treatment. After 120 minutes of gavaging with glucose, the sera from the mice were collected. Then, glucose in serum was measure by spectrophotometric analysis.

3.4.1 Spectrophotometric Analysis

The spectrophotometric analysis was carried out according to the instruction for glucose measurement (Cromatest, Linear Chemicals, S.L., Barcelona, Spain). This measurement was based on the Trinder reaction (Trinder 1969 and Barharn *et. al*, 1972). In principle, glucose was oxidized to D-gluconate by the glucose oxidase (GOD) with the formation of hydrogen peroxide. In the presence of peroxidase (POD), a mixture of phenol and 4-aminoantipyrine (4-AA) was oxidized by hydrogen peroxide, to from a red quinoneimine dye proportional to the concentration of glucose in the sample.

B-D-Glucose + H_2O+O_2 $\rightarrow$GOD$\rightarrow$D-Gluconate +H_2O_2----------(i)

4-AA + Phenol $\rightarrow\rightarrow\rightarrow H_2O_2 \rightarrow\rightarrow$POD $\rightarrow\rightarrow$Quinoneimine + H_2O-------(ii)

The reagent for serum glucose measurement contained **R1**(monoreagent) which containedphosphate buffer (100 mmol/ L , pH 7.5), glucose oxidase (10 KU/L), peroxidase (2 KU/L), 4-aminoantipyrine (0.5 mmol/L), phenol (5 mmol/L). The other reagent was **CAL** which contained Glucose Standard: Glucose (100 mg/dl ≈5.55mmol/L). This standard was organic matrix based.

The Monoreagent and the Standard were supplied as ready –to-use.

The procedure for determining the serum glucose level was as follows:

1. The reagents and the sample were brought to room temperature.

2. Pipetting of samples and reagents were preformed into labeled tubes according to Table below (Table-3.1).

Table 3.1 Mixtures of samples and reagents for serum glucose measurement

Tubes	Blank	Sample	Standard
Monoreagent	1.0 ml	1.0 ml	1.0 ml
Sample	-	10 µl	-
Standard	-	-	10 µl

3. The mixtures were mixed and the tubes were incubated for 10 minutes at room temperature or 5 minutes at 37°C.

4. The absorbance (A) of the samples and the standard were performed at 500 nm against the reagent blank. The colour was stable for about 2 hours if protected from light.

The amount of glucose was determined according to formula given below:

$(A_{Sample} / A_{Standard}) \times C_{Standard} = $ mg/dl. Glucose----------(iii)

Here, $A_{Sample} =$ Sample absorbance

$A_{Standard}$ = Standard absorbance

$C_{Standard}$ = Standard concentration

3.4.2 Statistical Analysis:

The data for animal experiment were expressed as mean ± SEM and were evaluated by Student's t-test to determine the significant difference between control groups and the experimental groups. The results obtained were compared with the control groups. The $p<0.05$ and 0.001 were considered to be statistically significant.

3.5 Evaluation of Analgesic Activity

Normally this method utilizes the response of albino mice against drug. The so called response is called writhing. When acetic acid is introduced, it generates pain in mice and they exhibit writhing. When they are treated with analgesic drug, pain sensation will be reduced and ultimately no. of writhing will be lowered. If the test drug becomes able to reduce the no. of writhing then it said to be of analgesic property, and if it is lowered than that of standard, then it is said to be of more active drug than that of standard.

The acetic acid induced writhing method (Whitle, 1964) is an analgesic behavioral observation assessment method that demonstrates a noxious stimulation in mice. The test consists of injecting the 0.8 % acetic acid solution intra-peritoneally and then observing the animal for specific contraction of body referred as 'writhing'. A comparison of writhing was made between positive control (Diclofenac sodium), control and test samples, which were administered orally. Positive control and test samples were administered orally 30 minutes prior to acetic acid injection. If the sample possesses analgesic activity, the animal that received the sample will give lower number of writhing than the control, i.e. the sample having analgesic activity will inhibit writhing.

3.5.1 Study design

Experimental animals were randomly selected and divided into six groups denoted as group-I, group-II, group-III, group-IV, group-V and group-VI, consisting of 8 mice in each group (Table 3.2). Each group received a particular treatment i.e. control, positive control and the four doses (50, 100, 200, 400mg/Kg body weight) of the extracts. Each mouse was weighed properly and the doses of the extracts and control reagents were adjusted accordingly.

Table.3.2 Experimental profile to assess the effects of extract of the samples on acetic acid induced writhing of mice.

Animal group	Treatment	No. of animal	Dose (/kg body wt)	Route of administration
I (Control)	0.7 ml acetic acid in 99.3 ml water	08	30 ml	Intraperitoneal
II (Positive control)	Aspirin (Diclofenac sodium)	08	250 mg	Oral
III (Test group)	methanol extract of plant sample	08	50 mg	Oral
IV (Test group)	methanol extract of plant sample	08	100mg	Oral
V (Test group)	methanol extract of plant sample	08	200mg	Oral
VI (Test group)	methanol extract of plant sample	08	400mg	Oral

3.5.2 Preparation of test sample

To prepare suspension of the test samples at different doses per body weight, 100 mg of samples was measured respectively. The extract was triturated in unidirectional manner by the addition of small amount of water to make a volume about 2 ml. For proper mixing of extract small amount of tween 80 was added to stabilize the suspension. For the preparation of diclofenac sodium at the dose of 200 mg/kg-body weight, 300 mg of diclofenac sodium was taken and a suspension in 1ml distilled water was made.

3.5.3 Methodology

Test samples, control and diclofenac sodium were given orally by means of a feeding needle. A thirty minutes interval was given to ensure proper absorption of the administered substances. Then the writhing inducing chemical, acetic acid solution (0.8 %) was administered intraperitoneally to each of the animals of a group. After an interval of 5 minutes, which was given for absorption of acetic acid, number of squirms (writhing) was counted for 15 minutes. Each mouse of all groups was observed carefully to count the number of writhing that they had made in 15 minute (Figure 3.1).

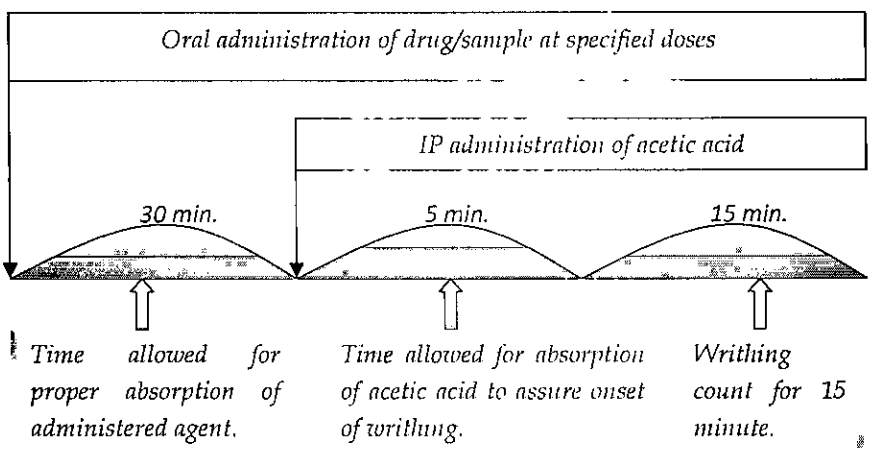

Figure 3.1: Schematic representation of acetic acid induced writhing of mice for analgesic activity study.

3.6 Brine Shrimp Lethality Assay for Cytotoxicity

Brine shrimp lethality bioassay is a recent development in the assay procedure for the bioactive compounds and natural product extracts, which indicates general toxicity as well as a wide range of pharmacological activities e.g. anticancer, antiviral, pesticides etc, (Anderson et al, 1988). Thus, in-vivo lethality of a simple zoological organism (brine shrimp "nauplii") can be used as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products (Hui et al, 1990). Natural product extracts, fractions or pure compounds can be tested for their bioactivity by this method.

3.6.1 Preparation of stock solution

100 mg of methanolic *S. caseolaris* extract was taken in 10 ml volumetric flask, dissolved in sea water adding with one or two drops of DMSO and volume was adjusted by sea water. The concentration of this solution was prepared 10 mg/ml & was considered as stock solution.

3.6.2 Preparation of sea water

38g sea salt (pure NaCl 20g and table salt 18g) was weighed accurately, dissolved in distilled water to make one liter and then filtered to get a clear solution.

3.6.3 Collection of brine shrimp

The brine shrimps were collected from shrimp cultivation farm. The shrimps were allowed to place in a tank containing sea water, and an air blower was attached to the lamp through the perforations in the dam.

3.6.4 Application of extracts and brine shrimp nauplii to the test tubes

10 clean test tubes were taken, 9 of which were for the samples in different concentrations and 1 for control experiment. Each test tube was accurately marked to indicate 10ml volume. Then 4ml of sea water was poured to each of the test tubes. Then with the help of the micropipette, specific volumes (0.1, 0.5,1. 10, 20, 40, 60, 80 & 100µl) of samples were transferred from the stock solutions to the test tubes to get final sample concentrations of 0.1, 0.5, 1, 10, 20, 40, 60, 80 & 100µl µg/ml respectively as the volume of each test tube will be 10 ml. To prepare the concentration of 0.1 & 0.5 µg/ml the stock solution was further diluted 10 times & from that 1 & 5 µl of samples was transferred to the marked test tubes. With the help of a pipette & magnifying glass 10 living shrimps were kept to each of the test tubes. Finally the volume of each test tube was adjusted with sea water up to the marked point (10ml). For the control, sea water and a small amount of alcohol (present at the conc. of 100 µg/ml sample solution) was taken in the control marked test tubes.

3.6.5 Counting of nauplii

After 24 hr the test tubes were observed and the number of nauplii survived in each test tube was counted and the results were noted. From this experiment, the percentage of lethality of brine shrimp nauplii was calculated at each concentration for each sample. The test was repeated twice.

4.0 Results and Discussion

4.0 RESULTS AND DISCUSSION

4.1 Determination of hypoglycemic activity of methanolic extracts of *S. caseolaris*

The present study was conducted to determine the hypoglycemic potential of methanolic extracts of fruits, leaves and barks of *S. caseolaris* L. in Swiss albino mice. Serum glucose level of the mice was determined spectrophotometrically at 500 nm in presence of different doses of methanolic extracts of *S. caseolaris* plant parts, standard drug Glibenclamide and controls. (Significant hypoglycemic activity of methanolic extract of fruits and barks were observed in doses determined based on the body weight of the mice (Table 4.1 and 4.3; Figure 4.1 and Figure 4.3). In these cases, methanolic extract of fruits showed stronger hypoglycemic activity in comparison to methanolic extract of barks. In case of methanolic leaf extract, the highest dose tested (400 mg/kg.body weight) showed the hypoglycemic result (Table4.2, Figure 4.2).

Table 4.1: Serum glucose levels of mice after 120 minutes glucose gavaging followed by oral administration of methanolic extracts of fruits

Sample no.	Control	Standard (Glibenclamide) (mg/dl)	Dose 50mg/kg (mg/dl)	Dose 100mg/kg (mg/dl)	Dose 200mg/kg (mg/dl)	Dose 400mg/kg (mg/dl)
1	100	30	115	87.5	62.5	87.5
2	120	100	120	120	85	72.5
3	160	65	105	117.5	75	87.5
4	150	75	115	77.5	127.5	105
5	145	85	112.5	105	100	112.5
6	112.5	97.5	95	85	115	67.5
7	150	92.5	110	117.5		107.5
8	160	97.5	112.5	85	120	100
Sum	1097.5	642.5	885	795	685	740
Mean±SD	137.18 ± 23.044	80.3125 ± 23.7335	110.625 ± 7.647	99.375 ± 17.5127	97.857 ± 24.513	92.5 ± 16.5219
SE		9.265	5.843	2.703	6.1916	8.148
T cal		4.862666	3.094	3.694	3.187	4.4566
Significance level%		0.1	1	1	1	0.1
5%significance level		Significant	Significant	Significant	Significant	Significant

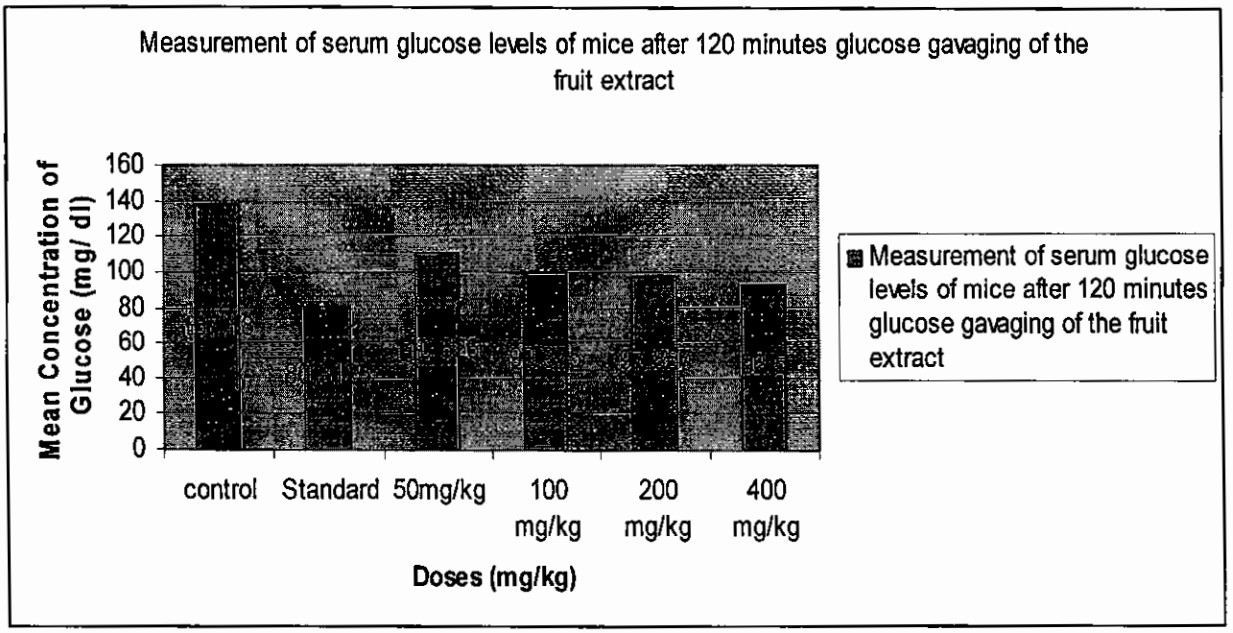

Figure 4.1: Serum glucose levels of mice after 120 minutes glucose gavaging followed by oral administration of methanolic extract of fruits.

Table 4.2: Serum glucose levels of mice after 120 minutes glucose gavaging followed by oral administration of methanolic extracts of leaves

Sample no	Control	Standard (Glibenclamide) (mg/dl)	Dose 50mg/kg (mg/dl)	Dose 100mg/kg (mg/dl)	Dose 200mg/kg (mg/dl)	Dose 400mg/kg (mg/dl)
1	105	68	120	135	98	80
2	110	98	115	121	120	72
3	145	70	125	115	88	85
4	150	80	145	98	121	100
5	145	75	136	108	128	111
6	114	110	98	110	134	65
7	155	91	150	115	100	101
8	165	100	112	160	123	90
Sum	1089	692	1001	962	912	704
Mean±SD	136.12± 22.93	86.5 ± 15.47348	125.125 ± 17.60225	120.25 ± 19.28545	114 ± 16.43168	88.0 ± 15.6022
SE	8.10739	5.47071	6.22336	6.818436	5.809475	5.51621
T- cal		5.073863	1.076262	1.498569	- 0.69772	4.907688
Significance Level %		0.1	-	-	-	0.1
5% Significance level		Significant	Insignificant	Insignificant	Insignificant	Significant

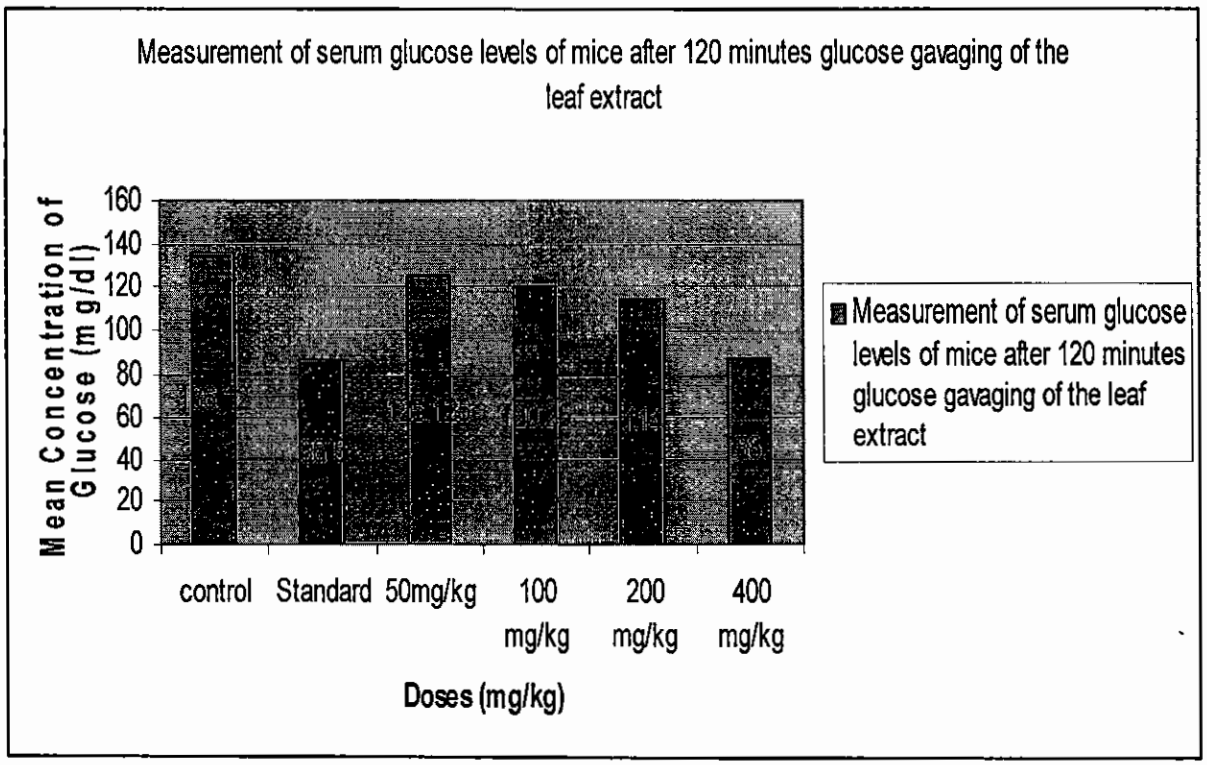

Figure 4.2: Serum glucose levels of mice after 120 minutes glucose gavaging followed by oral administration of methanolic extract of leaves

Table 4.3: Serum glucose levels of mice after 120 minutes glucose gavaging followed by oral administration of methanolic extracts of barks

Sample no.	Control	Standard (Glibenclamide) (mg/dl)	Dose 50mg/kg (mg/dl)	Dose 100mg/kg (mg/dl)	Dose 200mg/kg (mg/dl)	Dose 400mg/kg (mg/dl)
1	155	75	115	155	120	90
2	125	76	130	125	135	95
3	140	82	140	110	140	69
4	160	102	105	130	117	89
5	150	105	135	135	100	110
6	120	66	120	150	145	93
7	165	100	95	110	110	120
8	164	95	110	120	105	92
Sum	1179	701	905	1035	972	758
Mean ± SD	147.37± 17.3858	87.625 ± 14.68661	118.75 ± 15.52648	129.375 ± 16.78381	121.51 ± 16.75879	94.75± 15.11622
SE	6.14682	5.1925	5.489438	5.933975	5.925128	5.34439
T- cal		7.425625	3.473401	2.106805	3.030711	6.46078
Significance Level %		0.1	1	1	1	0.1
5% Significance level		Significant	Significant	Significant	Significant	Significant

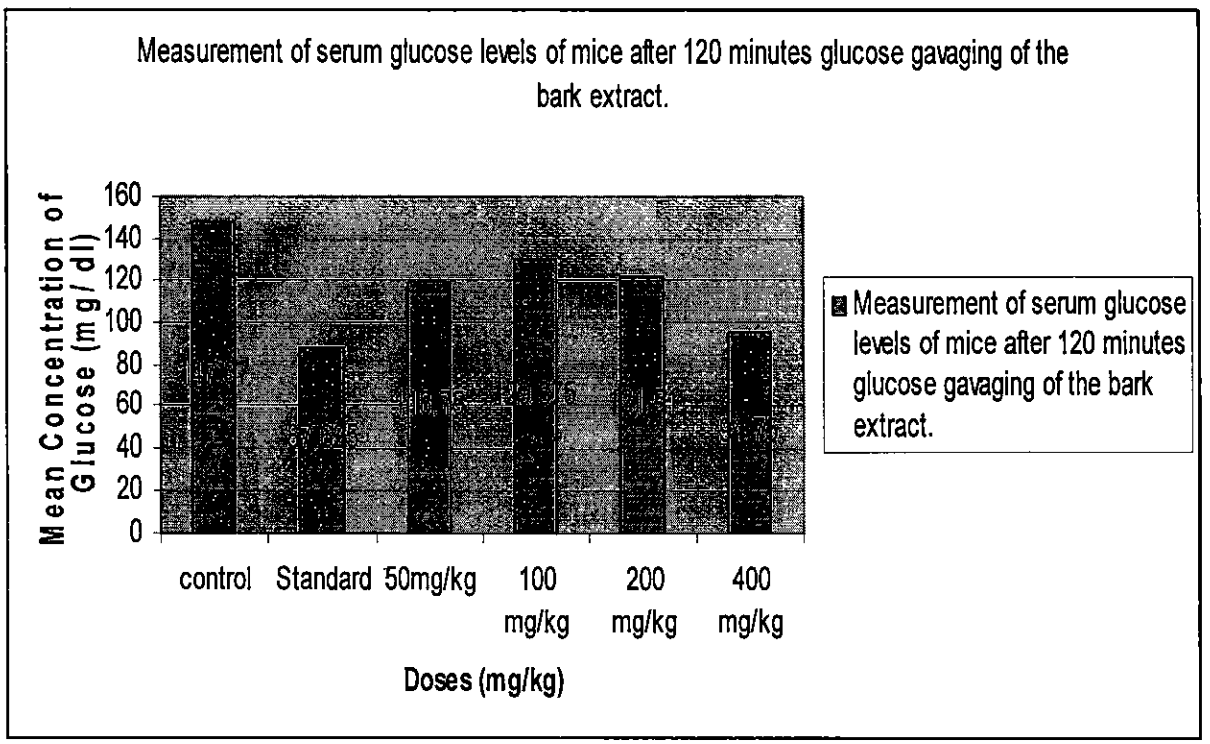

Figure 4.3: Serum glucose levels of mice after 120 minutes glucose gavaging followed by oral administration of methanolic extract of barks.

Oral administration of *S. caseolaris* leaf extract powder to mice led to insignificant results if investigated in dose-dependent manner whereas the extracts of fruits and barks extract showed significant results in a dose-dependent manner and the level of serum glucose decreased if compared to control groups with an exception at the 6^{th} dose from the extracts of leaves. The extracts from fruits and barks showed significant results in the doses investigated. At 6^{th} dose of both these extracts showed significant result ($p<0.001$) at the 400mg/kg- bw which was 94.75+15.11622 and 80.3125 ± 23.7335 respectively while other doses showed significant results at 1% level.

4.2 Evaluation of Analgesic Activity

Acetic acid-induced writhing test (Table 4.4, 4.5and 4.6) showed the effects of the methanolic extracts of *S. caseolaris* on acetic acid-induced writhing in mice (Figure 4.4, 4.5 and 4.6). At 6th dose (400mg/kg -bw) of leaf extracts of *S. caseolaris* showed significant reduction ($p<0.001$) of writhing induced by the acetic acid after oral administration in a dose dependant manner. The reference drug Aspirin (diclofenac sodium) was found more potent than the plant extracts at the entire dose levels.

Table 4.4: Acetic acid induced writhing of mice for studying analgesic activity with methanolic extract of fruits

Sample no.	Control	Standard (250 mg/dl	Dose 50mg/kg (mg/dl)	Dose 100mg/kg (mg/dl)	Dose 200mg/kg (mg/dl)	Dose 400mg/kg (mg/dl)
1	19	8	7	6	19	8
2	9	4	9	9	11	7
3	12	3	8	11	5	15
4	5	6	16	8	12	7
5	16	3	17	10	6	8
6	8	4	9	13	7	6
7	7	8	10	10	12	10
8	9	6	8	8	9	8
Sum	85	42	84	75	81	69
Mean ± SD	10.63±4.74906	5.25 ± 2.052873	10.5 ± 3.817254	9.375 ± 2.13391	10.125 ± 4.48609	8.625 ± 2.82527
SE	1.67906	0.7258	1.349603	0.754451	1.586072	0.99883
T- cal		2.938439	0.058026	0.679008	0.216476	- 0.6754
Significance level %		1	-	-	-	-
5% Significance level		Significant	Insignificant	Insignificant	Insignificant	Insignificant

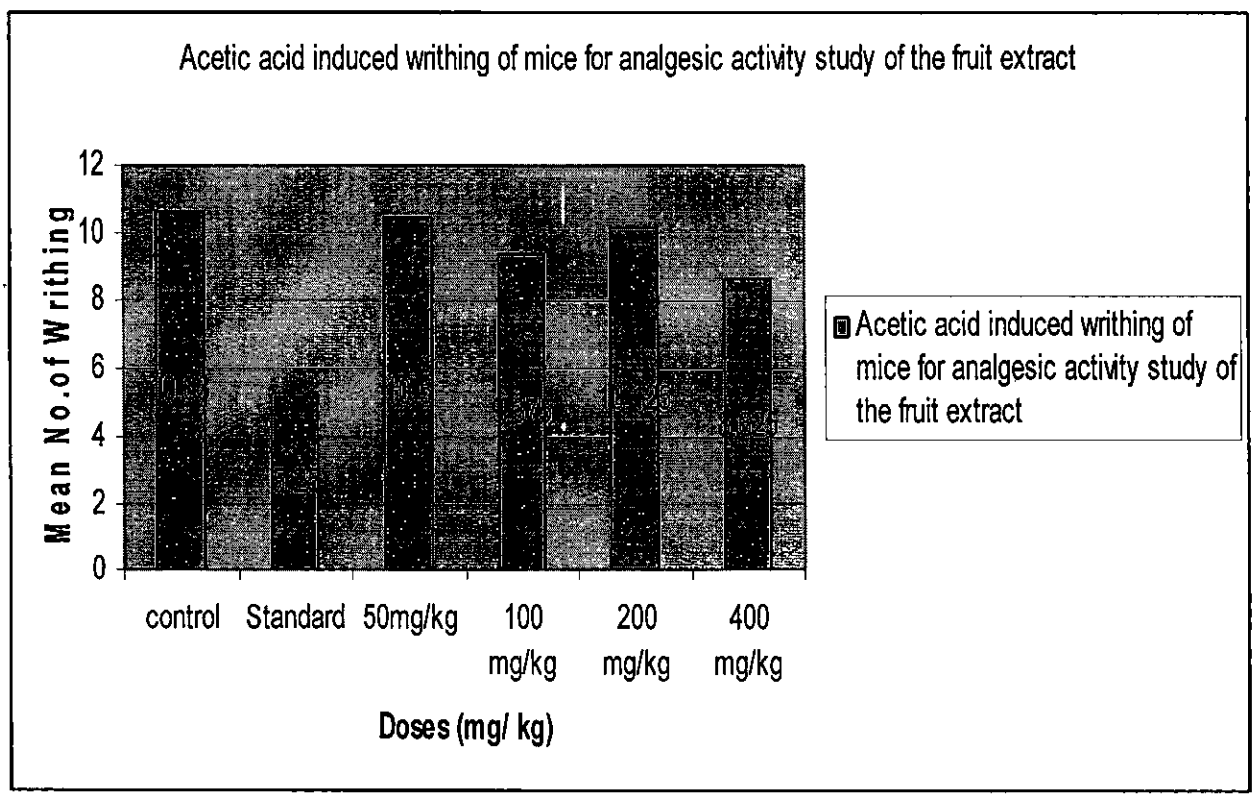

Figure 4.4: Acetic acid induced writhing of mice for studying analgesic activity with different doses of methanolic extract of fruits.

Table 4.5: Acetic acid induced writhing of mice for studying analgesic activity with different doses of methanolic extract of leaves.

Sample no.	Control	Standard (250 mg/dl	Dose 50mg/kg (mg/dl)	Dose 100mg/kg (mg/dl)	Dose 200mg/kg (mg/dl)	Dose 400mg/kg (mg/dl)
1	16	8	7	6	19	2
2	9	4	9	9	11	7
3	12	3	8	11	5	5
4	5	6	16	8	12	3
5	19	3	17	10	6	4
6	8	4	9	13	7	6
7	7	8	10	15	12	10
8	9	6	8	8	9	8
Sum	85	42	84	80	81	45
Mean ± SD	10.62 ± 4.74904	5.25 ± 2.052873	10.5 ± 3.817254	10.0 ± 2.9277	10.125 ± 4.48609	5.625 ± 2.66927
SE	1.67904	0.7258	1.349603	1.035098	1.586072	0.943729
T- cal		2.938439	0.058026	0.316862	0.216476	2.595933
Significance level %		1	-	-	-	1
5% Significance level		Significant	Insignificant	Insignificant	Insignificant	Significant

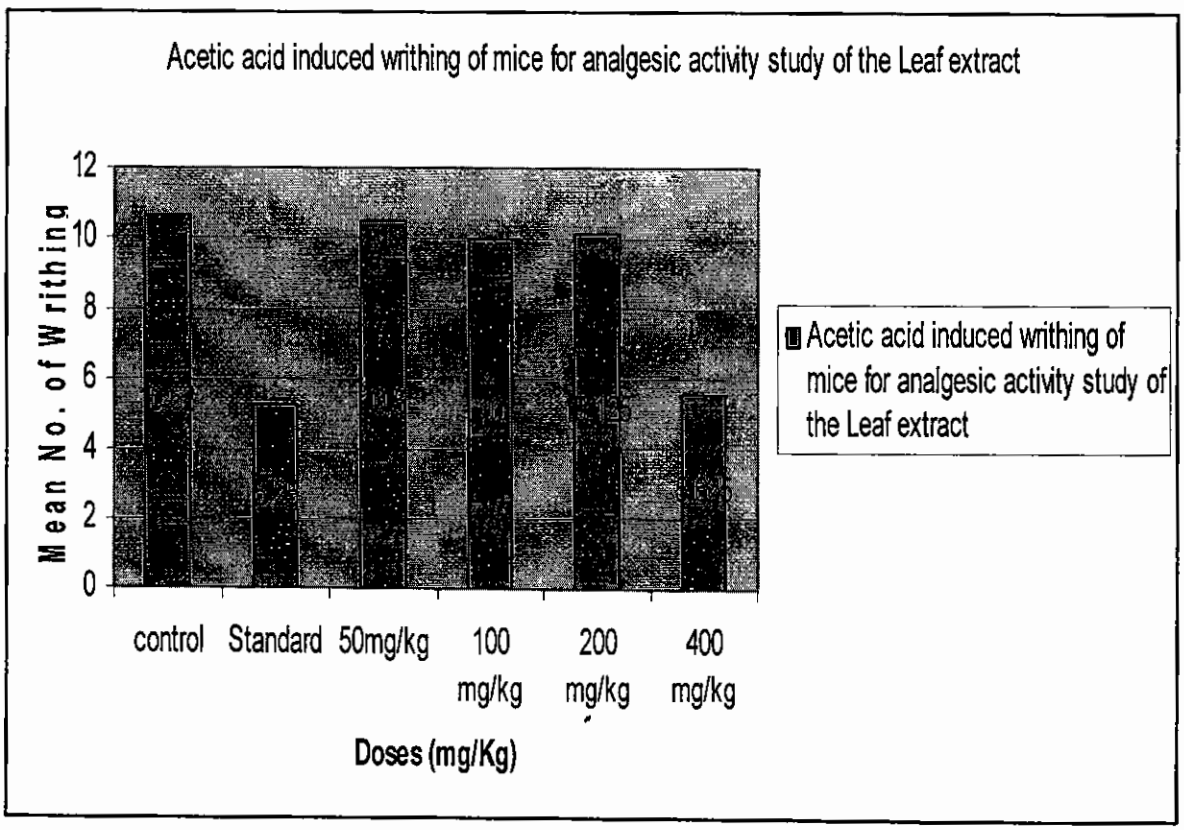

Figure 4.5: Acetic acid induced writhing of mice for studying analgesic activity with different doses of methanolic extract of leaves.

Table 4.6: Acetic acid induced writhing of mice for studying analgesic activity with different doses of methanolic extract of barks.

Sample no	Control	Standard (250 mg/dl)	Dose 50mg/kg (mg/dl)	Dose 100mg/kg (mg/dl)	Dose 200mg/kg (mg/dl)	Dose 400mg/kg (mg/dl)
1	4	5	6	4	17	8
2	7	1	7	8	4	5
3	12	7	13	12	5	4
4	5	4	8	5	12	7
5	13	1	18	7	5	4
6	14	5	4	10	6	6
7	8	9	6	9	8	8
8	8	6	8	9	9	9
Sum	71	38	70	64	66	51
Mean±SD	8.875 ± 3.72011	4.75 ± 2.764572	8.75 ± 4.559135	8.0 ± 2.61861	8.25 ± 4.399675	6.375 ± 3.696429
SE	1.31526	0.977424	1.611898	0.92582	1.55552	0.679745
T - cal		2.517272	-0.11993	0.54400	0.306817	-0.47599
Significance Level %		1	-	-	-	-
5% Significance level		Significant	Insignificant	Insignificant	Insignificant	Insignificant

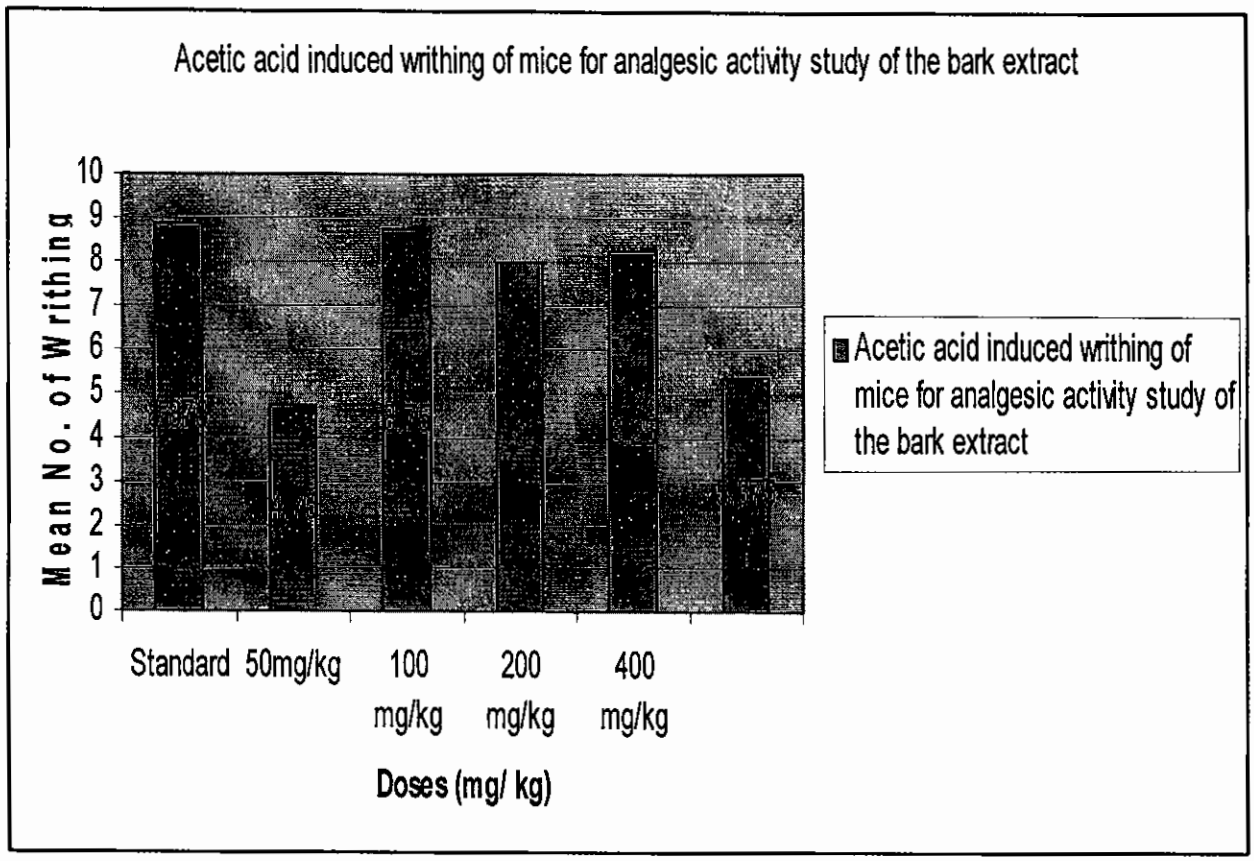

Figure 4.6: Acetic acid induced writhing of mice for studying analgesic activity with different doses of methanolic extract of barks.

In the acetic acid induced writhing test the methanolic leaf extract of *S. caseolaris* (400 mg/kg body weight) showed a significant (p<0.001) reduction in the number of writhes with 52.4 % of inhibition and fruit and bark extract were showed insignificant results in compared with control and standard. The constriction response of abdomen produced by acetic acid is a sensitive procedure for peripheral analgesic agents. This response is believed to be mediated by the prostaglandin pathways (Ronaldo *et al.*, 2000). The extract of *S. caseolaris* produced antinociceptive activity and thus indicates the presence of analgesic components that might influence the prostaglandin pathways. The present study on extract of *S. caseolaris* has demonstrated that this plant has significant analgesic activity and it justifies the traditional use of this plant in the treatment of various types of pains and inflammation.

4.3 Determination of cytotoxic activity using brine shrimp lethality bioassay

In this bioassay, the methanolic leaf extract of *S. caseolaris* showed lethality indicating the biological activity of the compound present in the extract. Test sample showed different mortality rates at different concentrations.

Table 4.7: Brine Shrimp lethality bioassay of *S. caseolaris* methanolic extract of leaf.

Group	Conc. (µgm/ml)	T-1	T- 2	Avg. no of alive shrimp (sample)	Avg. no of alive shrimp (control)	Std. Dev.	Std. err.	% mortaliy	Log conc.
Sonneratia caseolaris extract	5	7	6	6.5	10	0.5	0.20	35	0.698
	10	5	5	5		0	0	50	1.000
	20	3	4	3.5		0.5	0.20	65	1.301
	40	4	2	3		1	0.41	70	1.602
	80	3	2	2.5		0.5	0.20	75	1.903
	160	1	1	1		0	0	90	2.204
	320	0	0	0		0	0	100	2.505

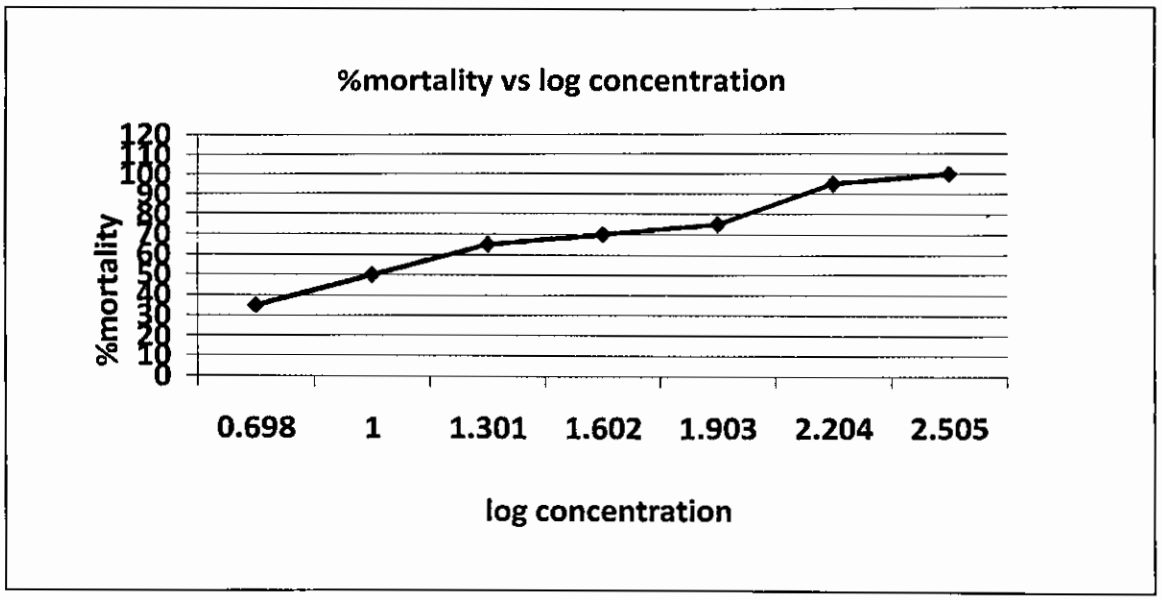

Figure 4.7: Percentage (%) mortality vs. log concentration of methanolic leaf extract of *S. caseolaris.*

Table 4.8: Brine Shrimp lethality bioassay using control drug Chloramphenicol

Group	Conc. (µgm/ml)	T-1	T- 2	Avg. no of alive shrimp (sample)	Avg. no of alive shrimp (control)	Std. Dev.	Std. err.	% mortaliy	Log conc.
Standard Chloramphenicol	5	9	9	9	10	0	0	10	0.698
	10	8	8	8		0	0	20	1.000
	20	5	4	4.5		0.5	0.20	55	1.301
	40	4	3	3.5		0.5	0.20	65	1.602
	80	2	1	1.5		0.5	0.20	75	1.903
	160	0	1	0.5		0.5	0.20	95	2.204
	320	0	0	0		0	0	100	2.505

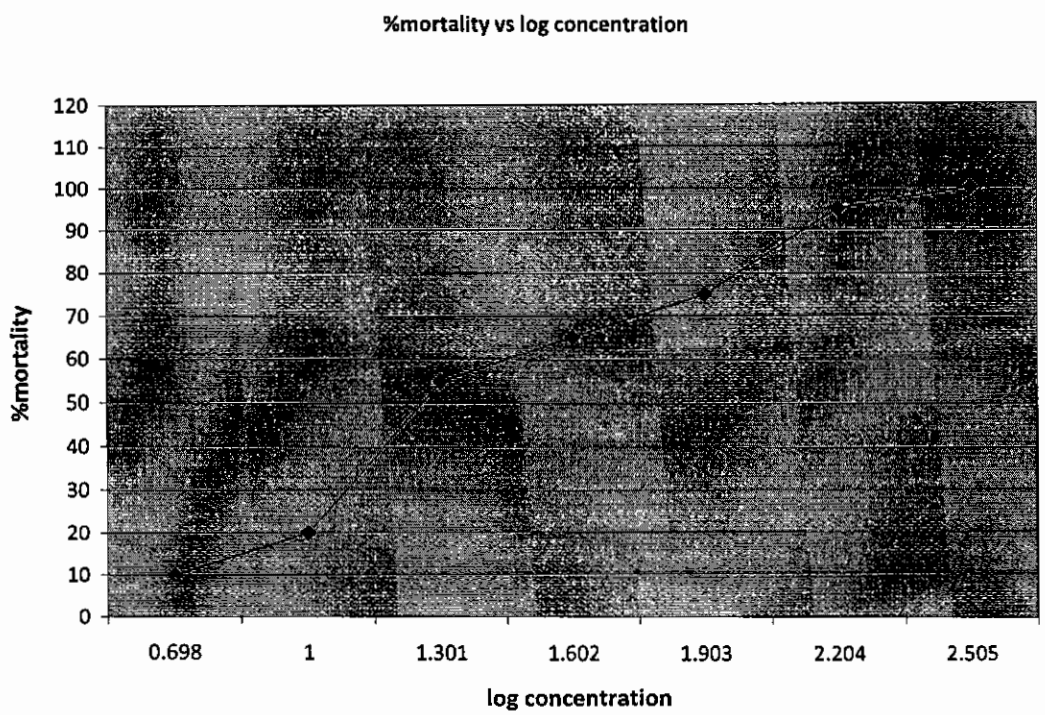

Figure 4.8: Percentage (%) mortality vs. log concentration in brine shrimp lethality bioassay using control Chloramphenicol.

The mortality rate of brine shrimp was found to increase with the increase in concentration of test samples (Table 4.7 and 4.8) and the Figure 4.7 shows the percentage of mortality against logarithmic concentration which showed an approximate linear correlation. From this figure the concentrations at which 50% mortality (LC_{50}) and 90% mortality (LC_{90}) of brine shrimp nauplii occurred were obtained by extrapolation & the values were be 10µg/ml & 160µg/ml respectively for methanolic leaf extracts of *S. caseolaris* . From Figure 4.8 shows the cLC_{50} and LC_{90} values of brine shrimp nauplii o were found to be 18.62µg/ml & 134.9µg/ml respectively for Chloramphenicol. The crude extracts were found to show strong lethality against the brine shrimp nauplii. These results tend to suggest its possible antitumor, antibacterial or pesticidal activities (Anderson et al, 1988). So from the study it is evident that methanolic leaf extract of *S. caseolaris* shows moderate cytotoxic effect. However, it is a preliminary investigation and further investigation is required to establish the potential cytotoxicity portfolio of this plant.

4.4 DISCUSSIONS:

The results obtained showed that the methanolic extract of *Sonneratia caseolaris* decreased blood glucose levels in glucose fed compared with their respective control groups. Glibenclamide caused significantly more hypoglycaemia in comparison with the plant extract. It has been reported that biguanide compounds produce hypoglycaemia in normal animals by reducing hepatic gluconeogenesis and by decreasing glucose absorption from gastrointestinal tract and also by increasing insulin sensitivity by increasing peripheral utilization of glucose. It is, therefore, conceivable that hypoglycemic principles in the extract exert effect probably by a mechanism similar to Glibenclamide.

In glucose tolerance test, the oral administration of *Sonneratia caseolaris* suppressed the increase in glucose level induced by glucose loading. Such an effect might be due to decrease in the rate of intestinal glucose absorption or by potentiation of pancreatic secretions or increasing the glucose uptake. It is known that the factors influencing the glucose metabolism under various physiological conditions do influence lipid metabolism as well (Jenkins et al., 1995). It has also been revealed that triglyceride accumulation increase considerably in diabetes mellitus (Iams and Wexler, 1977).

Hypercholesterolemia and hypertriglyceridemia have been reported to occur in diabetic (Riyad et al., 1988; Tarfa et al., 1988; Sharma et al., 1996) and a significant increase in cholesterol and triglyceride observed in our experiment in accordance to these studies. Reduction in the body weight in diabetic animals as well as in humans is well known. In case of diabetes, the body weight will increase when normal glycaemic levels is achieved which is particularly seen in sulfonyl ureas or insulin. *Sonneratia caseolaris* leaf extract increases body weight in glucose loaded mice, which might be due to increased insulin secretion and better glyceamic control.

The present study on pharmacognostical characteristics and preliminary phytochemical screening of *S. caseolaris* provide valuable information which may help in authenticating the genuine mangrove plant along with the nature of phytoconstituents present in it. The

above studies provide information regarding their identification and chemical constituents which may be useful for the standardization and preparation of monograph of *Sonneratia caseolaris*. The constituents of *S. caseolaris* may have several medicinal properties and can be utilized for the treatment of various diseases. Further research on this particular mangrove species may help in the isolation of therapeutically potent compounds which can be finally be subjected to pharmacological activities and clinical trials, thus leading to opening up new avenues in the use of natural products for therapeutic purpose. Sherine George et al., 2010 stated all of the results of bark powder coupled with the effect of chloform extract (bark) reveal that the bark of *Alstonia scholaris* possesses different significant pharmacological activities (Hypoglycemic, hyperlipidemic, anxiolytic & analgesic) at different doses. Shazid M. D. Sharker and Israt Jahan Shahid, 2010 reported ethanol extracts of the bark of *P. longifolia*, leaves of *E. serratus* and seeds of *T. ammi* showed significant antibacterial activity against some pathogenic bacteria and moderate to mild lethality to the brine shrimps tested. So in this study provides some scientific bases for the use of this plant as a remedy for stomach, skin and bacterial infections in folkloric medicine whose causative agents are some of the pathogens studies.

Brine shrimp lethality is a general bioassay, which is indicative of cytotoxicity, antibacterial activities, pesticidal effects and various pharmacologic actions (MacLaughi et al, 1991). The results indicate the ability of the plant extract to kill cancer cells in cell cultures, kill pests, and exert a wide range of pharmacologic effects (Meyer et al, 1982). The earlier report of Morton (1987) is that the extract could be used to treat cancer is also supported, by the present findings. The presence of saponins, alkaloids and cardiac glycosides may be responsible for the observed brine shrimps lethality activities of the extracts. The brine shrimps lethality further supports the antibacterial activities of *H. sabdariffa* on some pathogenic organisms observed in this study.

5.0 CONCLUSION:

In conclusion, the results suggests that different extracts of this mangrove plant (*S. caseolaris*) showed the hypoglycemic / antidiabetic activity in normal mice, improved glucose tolerance suggests that the extracts may stimulate the peripheral utilization of glucose by increasing insulin sensitivity to cells or by decreasing glucose absorption from gastrointestinal tract or by reducing hepatic gluconeogenesis and glycolysis, which is similar to that of glibenclamide. Although the present study gives some preliminary idea further study is necessary for the identification of the possible mechanism of action, isolation, purification and characterization of active components that may reveal new agents for diabetic therapy.

It is important to note that although the research with pure natural compounds delineates mechanism of their biological actions and help in development of new therapeutics, in traditional medicines they are present in mixtures and that mixture of compounds in traditional medicinal preparations are thought to be more effective than a single compound (Liu, 2005). The present study on pharmacognostical characteristics and preliminary phytochemical screening of *Sonneratia caseolaris* provide valuable information which may help in authenticating the genuine mangrove plant along with the nature of phytoconstituents present in it. The above studies provide information regarding their identification and chemical constituents which may be useful for the standardization and preparation of monograph of *S. caseolaris*. The constituents of the fruit, leaf and bark of *S. caseolaris* may have several medicinal properties and can be utilized for the treatment of various diseases. Further research on this particular mangrove species may help in the isolation of therapeutically potent compounds which can be finally be subjected to pharmacological activities and clinical trials, thus leading to opening up new avenues in the use of natural products for therapeutic purpose.

6.0 References

6.0 REFERENCES:

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7.0 Appendix

7.0 APPENDIX

Calculation of SD, SE, and t-value used in different biological studies:

$$SD=\sqrt{\frac{\sum(x-\bar{x})^2}{(n-1)}}$$

Where,

SD = *Standard Deviation*

x = *Value of individual observation*

$\bar{x}$ = *Mean value*

n = *Number of observations*

$$SE=\frac{SD}{\sqrt{n}}$$

Where,

SE = *Standard Error*

SD = *Standard Deviation*

n = *Number of observations*

t-value,

$$t'=\frac{\bar{x}-\bar{y}-\Delta_0}{\sqrt{\frac{s_1^2}{m}+\frac{s_2^2}{n}}}$$