Search for Beneficial Activities in Botanical Resources

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Dissertation is submitted as a part of the requirements for the degree of Doctor of Philosophy (Ph. D.)

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I accepted this thesis as confirming to the required standard for Ph. D.

Professor Hitoshi Aoshima ----------
(Ph. D. Supervisor) Hitoshi Aoshima

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DEDICATED TO

MY

BELOVED PARENTS

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SANZIDA MUBASSARA
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Abstract

As society ages and the amount of lifestyle-related stress increases, there is increasing demand for the development of supplements which improve physical and mental health. Therefore, it is important to find new botanical resources for use as active ingredients for supplements or drugs. Over 1000 of the estimated 5000 species of phanerogams found in Bangladesh, Southeast Asia are regarded as having useful chemical contents. A variety of these plants have been used traditionally as folk medicines. The chemical substances being extracted from many of these trees and fruits in Bangladesh, their biological activities were studied by both *in vitro* and *in vivo* test to clarify their beneficial effects on physical and mental health.

Antioxidative and antihistamine release activities together with total phenolic content in six extracts of Bangladeshi plants were measured in this study, since these beneficial activities have already been studied and found in some of twelve Bangladeshi fruits. The content of total phenolic in six crude extracts of mangrove samples were determined according to the Folin Ciocalteu procedure and the antioxidant activities of the extracts were evaluated by both DPPH method and reducing power test. The respective values showed some correlation (R-squared value = 0.95). The extract of *S. caseolaris* had the best antioxidative activities among the six mangrove samples with IC_{50} value of 18 μg/ml. All other plants used in this study also showed high antioxidative activity in relation to the total phenolic contents.

Then the extracts of six mangrove samples were used in anti-allergic experiments using rat peritoneal exudates cells (PECs). The effects on the release of histamine and leukotriene B_4 (LTB_4) from the PECs induced by Ca^{2+} ionophore, A23187 were measured by a fluorescence spectroscopy. All the mangrove samples were found to inhibit the release of both histamine and leukotriene B_4 (LTB_4) induced by the calcium ionophore A23187 from rat PECs, indicating the anti-allergic property. Among the mangrove samples, the most effective sample was *A. officinalis*. The IC_{50} value of this extract was estimated to be about 0.5 μM GAE. Since anti-hydrogen peroxide activity is found in citrus peels, their effects on the release of histamine from rat PECs and effusion of a dye into the intraperitoneal cavity of mice to study the anti-allergic activity of various kinds of citrus peels. All extracts of citrus peels examined inhibited histamine release and their inhibitions were enhanced when the extracts were heated at 100 °C for ten minutes. The oral administration of an aqueous extract of grapefruit peel suppressed inflammation in the cavity of rat as measured by dye effusion.

In order to characterize the active components, I performed the fractionalization of two samples, S. *caseolaris* and *A. officinalis* for their high anti-oxidative and anti-histamine activity respectively. Among the five fractions of *S. caseolaris*, ethyl acetate and ethanol fractions had very high antioxidative activities compared to other fractions. In case of *A. officinalis*, the chloroform fraction had the most activity, but some activity was also observed in the hexane and ethyl acetate fractions.

Ionotropic γ-aminobutyric acid receptor ($GABA_A$ receptor) is the main inhibitory neurotransmitter receptor in the brain. It is known that potentiation of the response of $GABA_A$ receptor induces the tranquilizing activity as the drugs such as benzodiazepine does. Administration of chemicals which potentiate the response of $GABA_A$ receptor is expected to prolong pentobarbital-induced sleeping time. Essential oils from cinnamon, coriander, clove and blended ones potentiated the response of the $GABA_A$ receptors expressed in *Xenopus* oocytes. Their effects on the pentobarbital induced sleeping time of mice were examined by both intraperitoneal and inhalational administration prior to intraperitoneal administration of pentobarbital, which is known to act on $GABA_A$ receptors. Co-administration of essential oils prolonged the pentobarbital-induced sleeping time in mice. These essential oils may modulate our mood through acting on $GABA_A$ receptors and induce relaxed feeling while inhalating these oils. To find out the extracts which may induce tranquilizing activity, $GABA_A$ receptors were expressed in *Xenopus* oocytes and effects of twelve extracts of Bangladeshi fruits and plants were examined electrophysiologically. The extracts of *S. caseolaris, T. bellirica, S. cumini and T. arjuna* significantly potentiated the response of $GABA_A$ receptors, though the extract itself induced no response of the receptors. Administration of these extracts prolonged pentobarbital-induced sleeping time in mice when they were administered both orally and intraperitoneally. Close relation (R-squared value = 0.92) was observed between the potentiation of the $GABA_A$ receptor response and the extension of the sleeping time. Both the potentiation of the $GABA_A$ receptor response and the extension of the sleeping time showed clear dose dependency of these promising samples.

In conclusion, the extracts of Bangladeshi mangrove plants have high polyphenol content, anti-oxidative and anti-histamine-release activity and are possibly useful to develop supplements good for human health. The extracts of citrus peels also inhibit the histamine release, suggesting the anti-allergic activity. Several extracts of Bangladeshi fruits and tree act on $GABA_A$ receptors both *in vivo* and *in vitro* as well as some essential oils. These extracts and essential oils may have potential regarding the development of a supplement with tranquilizing and sleep-inducing effects, which are beneficial for mental health.

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List of Abbreviations

ROS	Reactive Oxygen Species
DPPH	1, 1-Diphenyl-2-Picrylhydrazyl
TPH	Total Phenolic Compound
GAE	Gallic Acid Equivalents
OD	Optical Density
EtAc	Ethyl Acetate
EtOH	Ethanol
Chl	Chloroform
DW	Distill Water
BSA	Bovine Serum Albumin
PECs	Peritoneal Exudates Cells
LTB_4	Leukotriene B_4
CNS	Central Nervous System
IPSP	Inhibitory Post-Synaptic Potential
GABA	γ-Aminobutyric Acid
$GABA_A$	Ionotrophic γ-Aminobutyric Acid Receptors
IC_{50}	Half-maximal inhibition
K_1	Inhibition Constant
K_p	Dissociation Constant of GABA
V_m	Maximum Potentiation
ANOVA	Analysis of Variance
SD	Standard Deviation
R^2-value	Correlation Factor

CHAPTER ONE

GENERAL INTRODUCTION

1.1. Background of the research:

The plant kingdom is virtually food and drugstore. Though, now-a-days, the number of plants used in medicine has decreased but they are hidden away in various forms of medicines like pills, capsules and liquid drugs of which the active chemicals were originally found in plant kingdom. Plants not only continue to retain the historical significance as important sources of new drug but are also extremely useful as sources of "LEAD" compounds for structural modification and optimization in biochemical studies (Butlet, 2004). Thus medicinal plants have been subject of modern research in recent days for development of drugs and food supplements. Surveys show that 50% or more of all drugs in industrialized countries are natural products (James. 2003). For all these reasons, biologically active constituents (e.g., glycosides, steroids, terpenoids, alkaloids, flavonoids etc.) of plants have attracted the attention of chemists, biochemists, health scientists and biotechnologists.

More than 500 medicinal plants grown in Bangladesh have so far been enlisted and are used by traditional practitioners in the Indian sub-continent for treatment of various diseases like bronchitis, asthma, anaemia, diarrhoea, dysentery, typhoid, jaundice, rheumatism, haemorrhages, ulcers, gonorrhoea, hypertension, cardiac failure, cancer and many others (Goni, 2003). Some mentionable ones are *Embelica officinalis, Terminalia bellirica, Terminalia chebula, Terminalia arjuna, Adhatoda vasica, Allium sativum, Aloe sp, Alstonia sp, Andrographis paniculata, Asparagus racemosus, Atropa belladona, Azadirachta indica, Calotropis procera, Datura sp, Ephedra sp, Holarrhena antidysenterica, Jatropha sp, Momordica sp, Ocimum sp, Rouwalfia serpentina, Tinospora sp, Vinca sp,* etc. Some of the preparations of these medicinal herbs and plants are used as dietary supplements for the prevention and treatment of certain diseases, e.g., cardiovascular diseases, hypertension, diabetes, obesity, cancer etc.. Reportedly, dietary supplements and foods rich in polyphenols provide neuroprotection in adult animal models of ischemia and Alzheimer`s disease (Di Matteo and Esposito, 2003). Foods having high antioxidant are very essential for human health because of anti-aging and anti-carcinogenic activities. It has also been reported that some components of foods and drinks can act on receptors, channels, transporters and enzymes in the brain and modulate human consciousness. It has already found that many fragrant compounds in beverages and foods potentiate the response of ionotropic γ-aminobutyric acid receptors ($GABA_A$ receptors), while some catechin derivatives inhibit the response, using *Xenopus* oocytes expression system and an electrophysiological technique (Hossain *et al.*, 2007). It has also found that Na^+/glucose co-transporters are inhibited by some polyphenols such as catechin derivatives, which may be used for diabetic patients. So it is interesting to study the bioactive components of medicinal herbs and plants used for the preparation of traditional medicine or dietary supplements in Bangladesh.

1.2. Antioxidative activity:

An antioxidant is a substance or nutrient that significantly delays or prevents oxidation of an oxidisable substrate when it is present at a concentration lower than that of an oxidisable substrate (Dekkers *et al.*, 1996). It is manufactured within the body and can also be extracted from the human diet such as fruits, vegetables, seeds, nuts, meats and oils. On the other hand, free radicals produced as a by product of oxidation process, can start a chain reaction in the body and finally cause cell damage. Inside living cell, antioxidant defense system can prevent this reaction as they maintain complex system of multiple types of antioxidants such as vitamin A, C, E, selenium, carotenoids as well

as enzymes such as catalase, superoxide dismutase, various peroxidases. Normally a balance between the formation of radicals and defense exists in the body but when this system fails to cope with the production of excess free radicals especially reactive oxygen species (ROS), oxidative stress may occur. At that case, we need to take antioxidants in order to have the balance. So a balance between antioxidants and ROS is essential for normal living activities. If this balance is affected by any means like excess of ROS or shortage of antioxidants, the imbalance ultimately causes various disorders in living systems.

The antioxidaive activity is mainly due to its redox properties, which allow it to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Rice-Evans *et al.*, 1995). Plants often contain wide variety of antioxidaive molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinines and tannins), tocopherols, carotenoids and ascorbic acid (Cai *et al.*, 2004). These natural antioxidants are distributed in different parts of the plants such as leaves, bark, stems, wood, pods, fruits, roots, flowers, pollen and seeds (Chanwitheesuk *et al.*, 2005). Antioxidative compounds are thought to be beneficial to suppress ROS, which may cause aging or carcinogenesis. They also have many industrial uses such as preservatives in food and cosmetics. So, potential sources of antioxidaive compounds have been searched from several types of plant materials by using a number of different methods.

1.3. Anti-allergic activity:

Allergies are a damaging immune response and classified into four types. Among them, type 1 allergy is an immediate hypersensitive reaction produced by the normal immune system. The type 1 allergy plays an important role in reactions to food and environmental allergens (Younginger, 1992; Marks and marks, 1993). Mast cells play a crucial role in many physiological changes during an immediate allergic response through the production and release of chemical mediators such as histamine and eicosanoid (Kaliner *et al.*, 1982; Matsuo *et al.*, 2000; Takasugi *et al.*, 2008). So, compounds which inhibit the release of histamine and eicosanoid from mast cells will reduce allergic symptoms.

Allergy related research is now getting prevalence, as allergic disease has increased all over the world during the last two decades and it is assumed that the recent changes in environment are the main cause of this increase (Kawai *et al.*, 2005). For example, more than one-third of the population is now suffering from at least one of allergic diseases like asthma, topic dermatitis or allergic rhinitis in Japan. Though now half of the Japanese population has become sensitized with Japanese cedar pollens and now 24-29% of population is suffering from this disease (Kaneko *et al.*, 2005). Thus it is a central issue to reveal what environmental factor (s) cause such high prevalence and to find out strategies to prevent their development. Recently the change of diet is considered to be one of the environmental factors responsible for such increase. So an appropriate intake of foods and beverages with anti-allergic activities is expected to prevent allergic disease. Recently, anti-allergic activity has been found in foods such as yogurt or green tea. Reportedly, various polyphenols in foods and beverages have anti-allergic activities (Corvazier and Maclouf, 1985; Kanda *et al.*, 1998; Takasugi *et al.*, 2002; Takasugi *et al.*, 2008).

1.4. γ-Aminobutyric acid receptors and their expression in *Xenopus* oocytes:

γ-Aminobutyric acid (GABA) is the chief inhibitory neurotransmitter in mammalian central nervous system. It is a very popular compound present in various foods, since it is synthesized from glutamic acid by glutamate decarboxylase. Both

glutamic acid and GABA are essential for the overall balance between neuronal excitation and inhibition (Martin and Tobin, 2000). There are three types of the receptors that respond to the neurotransmitter GABA in the CNS i.e. $GABA_A$, $GABA_B$, and $GABA_C$ receptors. $GABA_A$ and $GABA_C$ receptors are ligand-gated chloride ion channels whereas $GABA_B$ receptors are metabotropic G-protein coupled ones.

$GABA_A$ receptors:

Various neurotransmitter receptors, especially the ionotropic γ-aminobutyric acid receptors ($GABA_A$ receptors) which are the main inhibitory neurotransmitter receptors in the human brain, are involved in defining mental state (Hossain *et al.*, 2007). These hetero pentamers are composed of various α, β, γ, δ and ε subunits that arrange circular formation and form a pore which transverses the membrane of the post-synaptic neuron.

They have a complex pharmacology, with binding sites for direct GABA agonists and antagonists together with multiple allosteric sites for benzodiazepine tranquillizers, for the barbiturate central nervous system depressants, for synthetic and endogeneous steroids, for general anesthetics and for ethanol (Cheibib and Johnston, 2000). By binding of GABA to the GABA binding sites of the receptors, the configuration of the receptors is changed, ultimately the central pore is opened, and the chloride ions rush into the postsynaptic neuron and causes initiation of an inhibitory post-synaptic potential (IPSP) that depresses excitatory depolarization, and thus decreasing the ability of the neuron to fire action potentials. If enough IPSPs have fired, these will stop the neuron from firing. When this inhibitory action occurs in many circuits at the same time, the activity of the amygdale as a whole decreases, and it fires less excitatory signals to other areas of the brain. This in turn leads to a reduction in the physiological and psychological markers of stress and anxiety. GABA thus has a calming, tranquilizing effect on our emotions and prevents us from becoming overwhelmed in stressful situations.

***Xenopus* oocyte expression system and electrophysiological measurements:**

The oocytes of *Xenopus laevis* have become popular for long-term electrophysiological measurements of the responses of the receptors or transporters expressed. Receptors and co-transporters are membrane-embedded protein molecules, which bind with a ligand and transport electricity. *Xenopus* oocyte expression system combined with a voltage-clamp electrophysiological method is a powerful technique for the expression of foreign genes of receptors, channels or transporters to characterize their functional properties (Buckingham *et al.*, 2006). Since *Xenopus* oocytes, which are round and have a diameter of > 1 mm, are larger, more stable, and simpler in shape than neurons or epithelial cells in small intestines, electrophysiological measurements of the receptors and transporters expressed in the oocytes can be made easily and repetitively for a long period. Moreover, the receptors composed of specific combinations of the subunits can be expressed and their pharmacological characteristics can be examined. This system is very useful to examine the effect of various compounds on receptors and transporters in brain and small intestines.

1.5. Objective of the study:

The purpose of this study is to screen out and identify promising samples which have antioxidative, anti-allergic and tranquilizing activities so that they can be used in the food, cosmetics and pharmaceutical industries to prepare functional foods, cosmetics and drugs with anti-allergic, antioxidative, tranquilizing and sleep-inducing activities. Therefore, the attempts could have been made to address the following:

1. To determine total phenolic contents of the methanolic or ethanolic extract of twelve Bangladeshi medicinal plants as well as to evaluate their antioxidaive activity by using DPPH and reducing power test.

2. To examine the effect of samples on the histamine release activity from the rat peritoneal exudates cell.

3. To study the effect of the crude extract on neural transmission through $GABA_A$ neuroreceptors by expressing the receptor in the *Xenopus* oocytes and electrophysiological measurement and to examine their cell toxicity of the extracts by measuring the non-specific currents and the membrane potential changes in non-injected oocytes.

4. To study the effect of the crude extract on sleeping-time in mice induced by sleeping drug pentobarbital.

5. To study the active component in the extracts by extraction with various solvents.

CHAPTER TWO

MATERIALS AND METHODOLOGY

2.1. Chemicals:

Folin-Ciocalteu's phenol reagent, fish gelatin, and histamine dihydrochloride were purchased from Sigma-Aldrich Co (St. Louis, MO). A-23187, bovine serum albumin (BSA), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Wako Pure Chemical Industry, Ltd., Osaka, Japan. Gallic acid was purchased from Nacalai Tesque, Kyoto, Japan. All of these chemicals and other reagents were of analytical grade.

Essential oil of *Eugenia caryophyllus* and *Coriandrum sativum* were purchased from Nippon-Ester Co., Ltd., Osaka and supplied from Promotool Co., Ltd., Tokyo. Sample A and B were blended by a perfumer and given from promotool Co., Ltd., Tokyo. Essential oil of the leaves of *Abies sachalinensis* was supplied from Forest Association of Shimokawa, Hokkaido and essential oil of the wood of *Cryptomenia japonica* and epi-cubinol were supplied from Kiku-Masamune Sake Braving Co., Ltd., Kobe **(Table 2)**.

2.2. Plant materials:

The twelve plants investigated in the present study and their traditional uses are presented in **Table 1**. Five of the samples were collected from the world's largest mangrove forest, in Bangladesh. The other six were collected from a local market in Khulna city, Bangladesh. They were all taxonomically identified by experts at the Bangladesh National Herbarium or authenticated at Forest and Wood Technology Discipline, Khulna University, Bangladesh. The plant materials were cut into small pieces and dried in the sun. The dried materials were ground into a powder with a grinder and stored separately in an air tight container in a cool, dark and dry place.

2.3. Preparation of extracts of the plants:

About 400 g of powdered material was placed in a clean, flat bottomed glass container (4 L) and soaked in 1.3 L of 80% ethanol for *Derris uliginosa*, *Sonneratia caseolaris, Embelica officinalis, Terminalia bellirica, Terminalia chebula, Terminalia arjuna, Syzigium cumini* and *Dillenia indica*. For *Avicennia officinalis*, *Hibiscus tiliaceous* and *Manilkara zapota*, 80% methanol was used instead of 80% ethanol. The container was sealed for a period of 7 days with occasional shaking and stirring. The whole mixture then underwent coarse filtration through a piece of clean, white cotton followed by filtration through Whatmann filter paper. The filtrate was concentrated using a rotary evaporator (Bibby RE200, Sterlin Ltd., UK) to obtain the crude extract. The sample yields were 12 to 15% (w/w). These crude extracts (20 mg) were dissolved in ethanol (1 mL) for experiments.

2.4. Preparation of aqueous extracts of citrus peels:

Grapefruit (*Citrus paradise* Mackfady.), orange (*Citrus sinensis* Osbeck), lemon (*Citrus limon* Burm.f) and lime (*Citrus aurantifolia* Christm.) were purchased from a local market in Yamaguchi, Japan **(Table 2)**. Citrus peels were removed without the pitch, using a knife, and minced into small pieces. The minced peels (5 g) were ground down in a mortar, and deionised water (5 ml) was added. After centrifugation at 12,000 rpm for 15 min at 4 °C, the supernatant was used for experiments. When the effects of boiling on the inhibition of histamine's release by citrus peels were examined, the aqueous extracts were kept at 1000 °C for 10 min before the measurements.

2.5. Preparation of fractions of the extracts:

One gram (1 g) of this extract was taken in an air tight bottle. Hereafter, hexane was added to prepare hexane fraction of the sample. After vigorous shaking and filtration through filter paper hexane was evaporated and the dried solid was taken as a hexane fraction (Hex). Precipitate on the filter paper was taken in a separate air tight bottle and chloroform was added. Chloroform fraction (Chl) was prepared by evaporation of chloroform after filtration through a filter paper. Subsequently following the same procedure as above, ethyl acetate, then ethanol, and at last distilled water was used to obtain ethyl acetate (EtOAc), ethanol (EtOH), and water fractions (DW). Twenty milligram (20 mg) of the fraction was dissolved in 1 ml ethanol or distilled water for conducting experiments.

For the preparation of pentane extract, 1 g of ethanolic extract was added in 40 mL of pentane followed by 24 hour vigorous shaking. After filtration through filter paper pentane phase was separated and the solvent was evaporated by an evaporator, and the solid was taken as pentane fraction. Finally the solid was dissolved in ethanol (20 mg/mL) and stored at 4 °C. The effect of these extracts on the GABA-elicited response of the bovine $GABA_A$ receptors was examined by the addition of the extracts to GABA solution to treat oocytes.

2.6. Determination of total phenolic compounds (TPH):

The total concentration of phenolic compounds (TPH) in the extracts was determined according to the Folin-Ciocalteu method (Vinson *et al.*, 2001) with gallic acid (GA) as the standard and expressed (mg) as gallic acid equivalents (GAE)/g of extract (Aoshima and Ayabe, 2007). One mL of diluted extract was mixed with 1 mL of Folin-Ciocalteu's reagent and vortexed for 5 s. Then, 1 mL of a 10% (w/w) sodium carbonate aqueous solution was added to the mixture. The mixture was incubated at room temperature for 1 h, after which colorimetric measurements were made at 700 nm. Each experiment was conducted three times.

2.7. DPPH radical scavenging activity:

The reaction mixture (total volume, 3 mL), consisting of 0.5 mL of a 0.5 M acetic acid buffer solution at pH 5.5, 1 mL of 0.2 mM DPPH in EtOH, and 1.5 mL of a 50% (v/v) ethanol aqueous solution with the fractions, was shaken vigorously (Blois, 1958; Aoshima *et al.*, 2004). After incubation at room temperature for 30 min, the amount of DPPH remaining was determined by measuring absorbance at 517 nm, and the radical-scavenging activity of each sample was expressed using the ratio of the decrease in absorption (%) relative to the control (100%) in the absence of the fraction. If the diluted fraction itself had an absorption value that was more than 1% of the control, it was subtracted from the value for the sample reaction mixture. That is, the radical scavenging activity (%) = 100 (A – B)/A, where A and B were the absorption of the control and the corrected absorption of the fraction reaction mixture, respectively (Aoshima *et al.*, 2004). Mean values were obtained from triplicate experiments.

2.8. Reducing power:

The reducing power of the extracts was determined according to the method of Oyaizu (1986). Phosphate buffer (2.5 mL, 0.2 M, pH 6.6) containing 0.4 mg/mL of extract was prepared. Then it was added to 2.5 mL of 1% (w/w) potassium ferricyanide, and mixed. After incubation at 50 °C for 20 min, the mixture was mixed with 2.5 mL of 10% (w/w) trichloroacetic acid and centrifuged at 650g for 10 min. The supernatant (2.5

mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride. Then the absorbance of this solution was measured at 700 nm. One mM potassium ferrocyanide in the buffer solution, which is produced from potassium ferricyanide by reduction, produced absorbance of OD 0.985 at 700 nm in a cell with a 1-cm long light path. Ascorbic acid (40 µg/mL phosphate buffer) served as a positive control.

2.9. Preparation of rat peritoneal exudate cells (PECs):

Male SD rats (8 weeks old) were purchased from Kyudo Co., Ltd., Tosu, Japan. Twenty milliliters of Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 12 mM $NaHCO_3$ and 5.6 mM glucose, p^H 7.4) containing 0.1% (w/w) BSA was injected into the peritoneal cavity. After the abdomen was gently massaged for 2 min, the cavity was opened, and the fluid containing the peritoneal exudate cells (PECs) was collected with a Pasteur pipette. The cells were gently washed with Tyrode buffer and then centrifuged at 200 x g for 10 min at 4 °C. To remove contaminating erythrocytes by hypotonic lysis, the cell pellets were resuspended in a modified ammonium chloride buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 10 mM EDTA.2Na, pH 7.4) and incubated for 5 min at 4 °C. The cell suspension was then centrifuged at 200 x g for 5 min at 4 °C and the cells were resuspended in the Tyrode buffer at 2 x 10^6 cells/mL. Cell viability was measured by trypan blue staining and mast cells were identified by toluidine blue staining (Takasugi *et al.*, 2002). The cell viability of this preparation was more than 95% and the proportion of mast cells was 5-10% of all the cells (Matsuo *et al.*, 2000).

2.10. Measurement of the inhibition of histamine release:

Rat peritoneal exudate cells (PECs; 500 µL, 2 x 10^6) were suspended in 48 µL of 25 mM $CaCl_2$, 12 µL of various concentrations of samples and/or 120 µL of 5 µM A23187 solution, and then the volume was adjusted to 1.2 mL with tyrode buffer, and incubated for 20 min at 37 °C. The reaction was terminated by incubating for 5 min at 4 °C. The cell suspension was then centrifuged at 300 x g for 10 min, and the amount of histamine in the supernatant was measured (Takasugi *et al.*, 2002).

The histamine content was measured by fluorometric assay (Shore *et al.*, 1959). The percent inhibition of histamine release was calculated with the following formula: inhibition of histamine release (%) = 100 {(positive control – histamine release with extract and A23187) / (positive control – negative control)}. The negative control was the histamine content without stimulation by A23187. The positive control was that after stimulation by A23187. All results were expressed as the mean ± SD of at least four determinations (n = 4).

2.11. Anti-noceceptive activity:

Experimental animal:

Male ddY mice aged 4-5 weeks, average weight 20-25 g.

Experimental animals were randomly selected and divided groups denoted as group-I, group-II, test group-I, test group-II, test group-III and test group-IV etc consisting of few mice in each group.

Each group received a particular treatment i.e. group-I for control, group-II for positive control, test group-I for extract at the dose of 200 mg/kg, test group-II for at the dose of 400 mg/kg, etc. Each mouse should weighed properly and the doses of the test samples and control materials should adjusted accordingly.

To prepare suspension of the test samples at the doses of 400 mg/kg per body weight, 400 mg of samples were measured by using electric balance. The extract was

triturated in unidirectional manner by the addition of small amount of tween-80. After proper mixing of extract and tween-80, the volume was adjust with distilled water in a 10 mL volumetric flask. Similarly, 200 mg of samples were mixed with distilled water in a 10 mL volumetric flask to make the test samples at the doses of 200 mg/kg per body weight; it was shaken well. For the preparation of Diclofenac sodium at the dose of 25 mg/kg body weight, 25 mg of Diclofenac sodium was taken and volume was adjusted in a 10 ml volumetric flask.

Tabulation of writhing:

The extract and the reference drug were administered orally 30 min before the administration of 0.7% acetic acid in a volume of 10 mg/kg intraperitonealy. Control mice received 1% tween-80 in water under the same experimental conditions. Immediately after injection of acetic acid each mouse of all groups was observed carefully to count the number of writhing that they had made in 15 min. The animal do not always perform full writhing, because sometimes the animals begin to produce writhing but they do not complete it. This incomplete writhing may be taken as half-writhing, so two half-writhing were taken as one full writhing. The percentage inhibition was calculated using the following ratio:

Percentage of inhibition= 100(Control mean-Treated mean)/(Control mean)

2.12. Preparation of cRNA and *Xenopus* oocytes:

The cRNA of the α_1 and β_1 subunits of bovine $GABA_A$ receptors were synthesized from cloned bovine $GABA_A$ receptor cDNA with RNA polymerase (Promega, Madison, WI) according to the manufacturer's instructions. The cloned cDNA was provided by Prof. Eric A. Barnard at the Medical Research Council Center, London, UK.

Adult female frogs (*Xenopus laevis*) were purchased from Hamamatsu Seibutsu (Kyozai Co., Hamamatsu, Japan). The oocytes were dissected from adult frog ovaries that had been kept in ice for 1 h. They were manually detached from the inner ovarian epithelium and follicular envelope after incubation in a collagenase (type I, 1 mg/mL; Sigma) solution for 1 h according to the procedure of Kusano *et al.* (1982). The oocytes were microinjected with cRNA in sterilized water and then incubated in modified Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$ and 0.41 mM $CaCl_2$ in 5 mM Tris at pH 7.6) containing 25 mg/L penicillin and 50 mg/L streptomycin at 15 to 18 °C for 2 to 7 days before electrophysiological measurements (Aoshima *et al.*, 2001).

2.13. Electrophysiological measurements of the response:

The membrane current of the receptors evoked by GABA was measured by the voltage clamping method with a voltage clamp amplifier (TEV-200A, Dagan Co., Minneapolis, MN) according to the procedure described in a previous paper (Mitou *et al.*, 2008). To examine the agonistic activities of the extracts alone, 40 μg/mL of each extract was dissolved in normal frog Ringer's solution (115 mM NaCl, 1 mM KCl and 1.8 mM $CaCl_2$ in 5 mM Tris at pH 7.2) were applied to the oocytes expressing $GABA_A$ receptors. Responses induced by 20 μM GABA were taken as a positive control. To examine the effect of the extracts on the GABA-elicited responses of the $GABA_A$ receptors, GABA dissolved in normal frog Ringer's solution with or without the extract being tested was applied to oocytes expressing the $GABA_A$ receptors. The respective solution was selected by switching a valve in the flow system and the electrical responses induced by a mixture of 10 μM GABA and 40 μg/mL of extract were measured. The control response was obtained by perfusing a 10 μM GABA solution without extract and was taken as 100%. Ethanol at high concentrations potentiates the response of $GABA_A$ receptors but the effect

of EtOH present in the extracts is negligible (Aoshima *et al.*, 2001). The measurement was repeated several times in the same oocyte and control values were recorded after every two or three measurements. Values were expressed as the mean of four experiments. Student's *t*-test was used to evaluate the significance of differences between the mean values of the sample and those of the control.

The response (%) was analyzed with the assumption of a simple equilibrium between the active compound and the receptor:

Response - 100 = $(V_m - 100)$[compound]/$(K_p$ + [compound])

where [compound], K_p and V_m are the concentration of the compound, the dissociation constant and the maximum potentiation of the receptors when all the receptor potentiation sites are occupied by the compound (Aoshima *et. al.*, 2001).

2.14. Measurement of pentobarbital-induced sleep in mice:

Male ddY mice aged 4 weeks and weighing 15 to 30 g were purchased from Kyudo Co., Ltd. (Tosu, Japan). They were housed in Plexiglas cages (10 mice/cage) with a stainless-steel mesh top and excelsior bedding (Clea Japan, Tokyo, Japan). Commercial solid (Clea Japan) and tap water were available ad libitum. The cages were placed in a room artificially illuminated by fluorescence lamps on a 12L:12D schedule (light period: 07:00-19:00), at a temperature of 25 ± 1°C(Umezu, 1999). All experiments proceeded in accordance with the guidelines of the Ethics Committee for Experimental Animals of the Yamaguchi University, Japan, which essentially follows the National Institute of Health Guide for Care and Use of Laboratory Animals.

Pentobarbital-induced sleep was measured as reported by Koda *et al.* (2003). In the present study, two types of extract administration were performed: intraperitoneal injection and oral administration. In the case of intraperitoneal injection, pentobarbital was dissolved in a physiological solution of sodium chloride. Body-weight was measured with a weighing scale. The extracts (10-100 mg/kg) were dissolved in olive oil and administered to mice intraperitoneally 30 min before intraperitoneal injection of pentobarbital (50 mg/kg). Olive oil without the extract was administered intraperitoneally as a control. The volume of sample injected was 1 mL/100 g (or 0.2 mL/20g mouse). Oral administration of the extracts was performed by suspending water supply overnight followed by provision of ad libitum access to water containing the extract (2 mg/mL) 5 hours prior to the administration of pentobarbital. Average administration dose of the extracts was estimated from the volume of water with extract consumed. Sleeping time was measured as the time between disappearance and recovery of the righting reflex. To examine the effect of the extracts alone on the behavior of mice, the extracts (100 mg/kg) dissolved in olive oil were administered to mice intraperitoneally and the behavior observed for 2 h.

Table-1: Plant samples and their uses[a].

Local name	Name of the plant	Family name	Part used	Medicinal use
White mangrove (Kala Baen)	*Avicenia officinalis* Linn	Avicenniaceae	Leaves	Anti-allergy and diet.
Derris (Pan lota)	*Derris uliginosa*	Leguminosae	Leaves	Anti-allergy and fish poison.
Beach hibiscus	*Hibiscus tiliacious* Linn	Malvaeceae	Leaves and Stems	Scorpion-sting and snake-bite.
Sapodilla (Sofeda)	*Manilkara zapota* (L.) Royen	Sapotaceae	Bark	Tonic and febrifuge.
Mehogoni	*Swietenia mahagoni*	Meliaceae	Bark	Tonic and aphrodisiac.
Mangrove apple (Orali)	*Sonneratia caseolaris* Linn	Sonneratiaceae	Leaves	Astringent and antiseptic.
Amla (Amloki)	*Embelica officinalis* (Gaertn.)	Euphorbiaceae	Fruits	Astringent, diuretic and laxative.
Black Myrobalan (Horitoki)	*Terminalia chebula* (Gaertn.) Retz.	Combretaceae	Fruits	General tonic, astringent and purgative.
Arjuna Myrobalan (Arjun)	*Terminalia arjuna* Roxb	Combretaceae	Bark	Cardiac tonic, astringent and febrifuge.
Beleric Myrobalan (Bohera)	*Terminalia bellirica* Roxb	Combretaceae	Fruits	Laxative, astringent and tonic.
Black berry (Jam)	*Syzygium cumini* Linn	Myrtaceae	Fruits	Diet, diarrhea and ring warm.
Elephant apple (Chalta)	*Dillenia indica* Linn	Dilleniaceae	Fruits	Astringent and pain killer.

[a] The Botanical sources and uses were collected from Goni (2003) and Balasooriya *et al.*, (1982)

Table 2: Various Citrus species and essential oils with their uses.

Local name	Name of the plant	Family name	Part used	uses
Orange	*Citrus sinensis* Osbeck	Rutaceae	Peels of fruit	Flavouring agent for perfume, cleaning product and aroma oil.
Grapefruit	*Citrus paradise* Mackfady.	Rutaceae	Peels of fruit	Oil from peels are used in aromatherapy
Lime	*Citrus aurantifolia* Christm.	Rutaceae	Peels of fruit	Peel oil is used in aromatherapy and dried peels are used as flavouring agent
Lemon	*Citrus limon* Burm.f	Rutaceae	Peels of fruit	Peel oil is used in aromatherapy and dried peels are used as flavouring agent
Clove oil	*Egenia caryophyllus*	Myrtaceae	Flower buds	Aromatherapy and also culinary purpose.
Coriander oil	*Coriandrum sativum*	Apiaceae	Seed	Aromatherapy and also culinary purpose.
Sample A	Mixture of 20% *Coriandrum sativum,* 50% *Juniperus virginiana* , 20% *Abies balsamea* and 10% *Cinamomum camphora*	Apiaceae Cupressaceae Pinaceae Lauraceae	Blended one	Aromatherapy
Sample B	Mixture of 70% *Jasminum sativum,* 45% *citrus reticulata,* 45% *Cidrus atlantica* and 3% *Egenia caryophyllus*	Oleaceae Rutaceae Pinaceae Myrtaceae	Blended one	Aromatherapy
Conifer sp.	*Abies sachalinensis* Masters,	Pinaceae	Leaves	Aromatherapy.
Japanese ceder	*Cryptomenia japonica*	Cupressaceae	Wood	Aromatherapy.

CHAPTER THREE

ANTIOXIDATIVE AND ANTI-ALLERGIC ACTIVITY

3.1. Antioxidaive and anti-allergic activity of some mangroves in Bangladesh.

INTRODUCTION

Antioxidative compounds are reported to suppress the reactive oxygen species (ROS). The pathogenesis of diseases like cardiovascular disorders, cancer, aging, inflammation, and brain dysfunction is accompanied by the production of free radicals leading to oxidative stress. Antioxidants are used in food industry and pharmaceuticals as additives. Widely used synthetic antioxidants are now under question due to their side effects like carcinogenicity (Gulcin *et el.*, 2007). Therefore in response to the growing consumer concern, search for antioxidants and/or antioxidant principles from natural sources have gained interest and many plants reportedly have potential antioxidative activity (Pokorny, 1991; Banerjee *et el.*, 2008). Phenolic compounds, which are secondary metabolites in plant materials, are known to be responsible for antioxidative effect. Fruits and vegetables are the main sources of phenolic compounds of human diet. Other sources, such as grain, herbs and spices, also have received particular attention as sources of antioxidants (Hannum, 2004; Nakatani, 2000).

In early spring, many people suffer allergic reactions to cedar pollen in Japan. Allergies are a damaging immune response and classified into four types. Type 1 plays an important role in reactions to food and environmental allergens (Younginger, 1992; Marks and marks, 1993). Mast cells play a crucial role in many physiological changes during an immediate allergic response through the production and release of chemical mediators such as histamine and eicosanoid (Kaliner *et al.*, 1982; Matsuo *et al.*, 2000; Takasugi *et al.*, 2008). So, compounds which inhibit the release of histamine and eicosanoid from mast cells will reduce allergic symptoms. Reportedly, various polyphenols in foods and beverages have anti-allergic activities (Corvazier and Maclouf, 1985; Kanda *et al.*, 1998; Takasugi *et al.*, 2002; Takasugi *et al.*, 2008).

Bangladesh has the largest mangrove forests in the world which has been a vast source of bioactive compounds. Mangrove plants have been used in traditional folk medicines and extracts from mangrove species are widely used throughout the world. They provide a rich source of steroids, triterpenes, saponins, flavonoids, alkaloids and tannins (Mishra and Sree, 2007).

In a previous paper (Hossain *et al.*, 2008), Bangladeshi fruits were screened for antioxidative, anti-amylase, anti-glucosidase, and anti-histamine release activities. In this current study, six Bangladeshi mangrove plants, traditionally used as folk medicines have been screened for both anti-histamine and anti-leukotriene B_4 (LTB_4) release activity and antioxidative activity. The aim of this study is to find medicinal plants which can be used in the food, cosmetic and pharmaceutical industries to prepare functional foods, cosmetics, and drugs with anti-allergic or antioxidative activity.

RESULTS

Total phenolic (TPH) content, DPPH radical scavenging activity and reducing power:

The total phenolic (TPH) content, DPPH radical scavenging activity, and reducing power of the extracts of Bangladeshi plants were measured and tabulated in **Table 3**. The extract of *S. caseolaris* has much TPH and DPPH radical scavenging activity (IC_{50} = 18 μg/mL). Reportedly, the activity of antioxidants is concomitant with the development of reducing power (Duh *et al.*, 1999). As expected, this extract's reducing power is also large.

Among the six samples, the extract of *Sonneratia caseolaris* contained highest amount, *i.e.*, 20.39 mg GAE/g. *Switenia mahagoni* and *Deris uliginosa* contained 5.77 and 5.56 mg GAE/g respectively. Other three, *Avicennia officinalis, Hibiscus tiliaceous* and *Manilkara zapota* contained comparatively lower amount.

At 50 μg/ml concentration, extract of *Sonneratia caseolaris* showed the highest DPPH radical scavenging activity (87.7%) followed by *Avicennia officinalis,* (18.3%), *Hibiscus tiliaceous* (10%), *Switenia mahagoni*(7.15%), *Manilkara zapota* (3.8%) and *Deris uliginosa* (3.7%). Since S*onneratia caseolaris* exhibited strongest activity it dose-dependently scavenged the DPPH radical, with IC_{50} value of 18 μg/mL.

The reducing capability of a compound may serve as a significant indicator of its potential antioxidative activity. Here the most reducing activity comes from the *Sonneratia caseolaris* (O.D. 2.11) followed by *Switenia mahagoni* (O.D. 1.06). *Deris uliginosa, Avicennia officinalis, Hibiscus tiliaceous* and *Manilkara zapota* have poor acivity.

Generally, extracts with a higher phenolic content show more DPPH radical scavenging activity and reductive activity. Here we found a correlation (R-squared value=0.956348) among total phenolic content and DPPH radical scavenging activity **(Fig. 3. 2a.)**. We also examined the correlation between TPH content and the reducing power of all extracts, and obtained a correlation of 0.952939 **(Fig. 3.2b.)**.

Fractionation of the promising samples:

As it is necessary to clarify the component(s) of the extract responsible for beneficial activities, the extract was further extracted by various solvents like hexane (Hex), chloroform (Chl), ethyl acetate (EtOAc), ethanol (EtOH), and water (DW) fractions were prepared to study their polyphenol content, antioxidative and anti-histamine release activities.. The solvent of the fractions was evaporated by an evaporator, and the solid was dissolved in EtOH.

Among the five fractions of *S. caseolaris*, it was found that EtOAc and EtOH had very high antioxidative activities and their polyphenol contents were also high compare to other fractions **(Table 4)**.

Inhibition of histamine release:

We examined the effect of the extracts of Bangladeshi plants on the release of histamine from rat PECs. The PECs (1 x 10^6/mL) were stimulated with 5 μM A23187 for 20 min in the presence of the extracts. As shown in **Fig. 3.3**, all examined extracts except that of *S. mehagoni* inhibited significantly the release of histamine from the cells at the same concentration of polyphenols (2 μM GAE). The dose-dependence of the inhibitory effect of the extract of *A. officinalis* was examined and is shown in **Fig. 3.4**. As a preliminary experiment, the extract was further extracted by various solvents to

study the components which inhibit the release. The solvent of the fractions was evaporated by an evaporator, and the solid was dissolved in EtOH. Then, inhibition of the release of histamine by the extract in the fractions was measured as shown in **Fig. 3.5**. Most activity moved to the chloroform fraction, but some activity was observed in the Hex and EtOAc fractions.

Inhibition of LBT_4 release:

We examined the effect of the extracts of Bangladeshi plants on the release of leukotriene (LTB_4) from rat PECs. The PECs (1×10^6/mL) were stimulated with 5 μM A23187 for 20 min in the presence of the extracts. As shown in **Fig. 3.6**, all extracts examined inhibited significantly the release of histamine from the cells at the same concentration of polyphenols (2 μM GAE).

Effect of the plant extract on the acetic acid-induced writhing:

The results of the acetic acid-induced writhing test in mice are given in **Table 5**. At doses of 200 and 400 mg/kg, the *D. uliginosa* extract inhibited the writhing responses of mice caused by the intraperitoneal administration of acetic acid. At these doses, the average numbers of the writhes were lower than the control group. Here maximum inhibition was 61.7% with the dose 400 mg/kg. Reference drug, Diclofenac sodium showed 76.7% inhibition at a dose of 25 mg/kg.

DISCUSSION

Many of the health-promoting activities of fruit, such as anti-cancer, anti-diabetic, anti-mutagenic, anti-microbial, and anti-allergic effects, may be related to anti-oxidative activity. A relationship between TPH content and antioxidative and anti-allergic activities has already been reported (Yamada *et al.*, 1999). In a previous paper (Hossain *et al.*, 2008), we reported that extracts of some Bangladeshi fruits have beneficial properties such as antioxidative, anti-amylase, anti-glucosidase and antihistamine-release activity. The Bangladeshi mangrove plants examined in this study also have antioxidative and antihistamine-release activity, though they have no significant anti-amylase activity (data not shown). Here a correlation between TPH content and scavenging DPPH radical suggested that the level of scavenging activity of the extracts was closely related to their phenolic groups. A very high correlation between TPH content and the reducing power of these extracts also suggested that the antioxidative activities increase with increases in polyphenol content (Duh *et al.*, 1999). Antioxidants such as polyphenols are very important for the prevention of cardiovascular disorders, cancer, aging, inflammation and brain dysfunction (Ames, 1983; Ames *et al.*, 1993; Kris-Etherton, *et al.*, 2002; Shon *et al.*, 2004). So the extracts of these plants may be useful to develop supplements and cosmetics with antioxidative activity.

Notably, the extract of *S. caseolaris* has high TPH and strong DPPH scavenging activity (IC_{50} = 18 μg/mL). Its reducing power is also large. *S. caseolaris* is a small tree distributed in tidal creeks and mangrove swamps of Bangladesh. The fruit is used as a poultice, on sprains and swellings. The fermented juice of the fruit is useful in arresting hemorrhage and stop-bleeding treatment of piles (Kirtikar and Basu, 1987).

A type I allergy is an immediate hypersensitive reaction to, for example, food or environmental allergens (Younginger, 1992; Marks and Marks, 1993). Mast cells play a crucial role in the pathogenesis of this type of allergy through the production and

release of chemical mediators such as histamine and eicosanoid (Matsuo *et al.*, 2000; Takasugi *et al.*, 2008), which trigger various pathophysiological events in the acute phase of the reaction, including an increase in vascular permeability, the contraction of bronchial smooth muscle or production of mucus, and neutrophil chemotaxis (Kaliner *et al.*, 1982; Robinson and Holgate, 1985). Therefore, it is important to inhibit the release of mediators for the prevention and/or alleviation of allergic symptoms. All extracts examined inhibited the release of both histamine and LTB_4 from PECs (**Fig. 3.3** and **Fig. 3.6**). Reportedly, gallic acid, which is very ubiquitous in mangrove trees, inhibits histamine release from mast cells, mediated by the modulation of cAMP and intracellular calcium (Kim *et al.*, 2006). The polyphenols in mangrove extracts possibly play an important role in the suppression of histamine and LTB_4 release (Corvazier and Maclouf, 1985; Kanda *et al.*, 1998; Takasugi *et al.*, 2002; Takasugi *et al.*, 2008). From the dose-inhibition relationship, the IC_{50} value of *A. officinalis* was estimated to be about 0.5 μM GAE from **Fig. 3.4**. The mangrove tree, *A. officinalis*, commonly known as Baen or Kala baen in Bangladesh, is a tall (25 m) tree widely distributed in coastal forests in Southeast Asia. The seeds, barks, leaves and fruits of this tree are used as food or folk medicine. The barks and leaves are used in the treatment of asthma, diabetes, and rheumatism (Balasooriya *et al.*, 1982). The extract of this tree may be of use for the development of supplements, cosmetics and drugs with anti-allergic activity. Since extracts of other mangroves also inhibited the release of both histamine and LTB_4, further experiments are necessary to clarify the component(s) of the extracts responsible for these activities. It is also necessary to examine whether they reduce the type I allergy when administered to humans. It is worth searching for other beneficial effects of these medicinal plants in future.

Acknowledgments: I want to acknowledge Dr. Mikako Takasugi in Department of Applied Chemistry and Biochemistry, Faculty of Engineering, Kyushu Sangyo University for measuring the release of LTB_4 from rat PECs and Ms. Tsujiyama Iyo of Yamaguchi University for her help during measurement of histamine release.

Table 3: Total polyphenolic content at 20 mg/mL, DPPH radical scavenging activity at 50 μg/mL, and reducing power at 0.4 mg/mL of six Bangladeshi plants.

Name of plant	Total polyphenol content (μM GAE)	DPPH radical scavenging activity (%)	Reducing power (OD)
A. officinalis	329 ± 14	18.3 ± 1.3	0.492 ± 0.028
D.uliginosa	556 ± 25	3.7 ± 0.8	0.433 ± 0.017
H. tiliacious	312 ± 7	10.0 ± 1.3	0.635 ± 0.056
M. zapota	222 ± 4	3.8 ± 3.8	0.421 ± 0.019
S. caseolaris	2039 ± 66	87.7 ± 0.2	2.11 ± 0.28
S. mahagoni	577 ± 11	7.15 ± 1.5	1.06 ± 0.040

One mM potassium ferrocyanide in the buffer solution, which is produced from potassium ferricyanide by reduction, produced absorbance of OD 0.985 at 700 nm in a cell with a 1-cm-long lightpath.

Table 4: Total polyphenolic content, DPPH radical scavenging activity and reducing power of the different fractions of *S. caseolaris* leaf extracts.

Samples	Total polyphenol (mg GAE/gm sample)	DPPH radical scavenging activity (%)	Reducing power (OD)
Hex fraction	4.31 ± 0.22	Nil	0.045± 0.019
Chl fraction	8.75 ± 0.27	25.59± 8.41	0.153 ± 0.013
EtOAc fraction	40.49 ± 0.69	89.15± 0.26	1.765± 0.037
EtOH fraction	37.12 ± 0.34	90.95 ± 0.6	1.356± 0.014
DW fraction	16.81 ± 0.34	46.65 ± 4.97	0.081 ± 0.02

GAE: Gallic acid equivalent, OD: optical density, Values are the mean of three replicates ± SD.

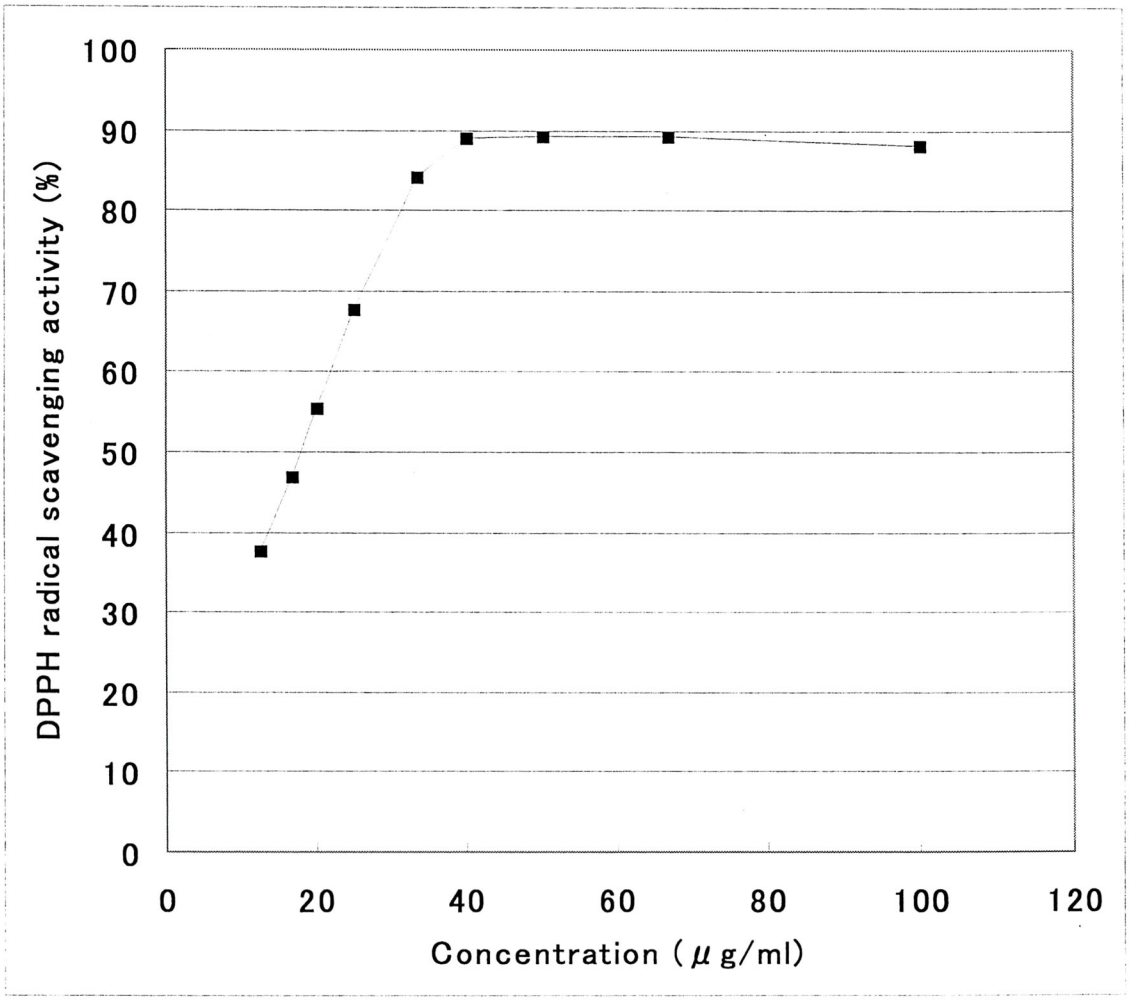
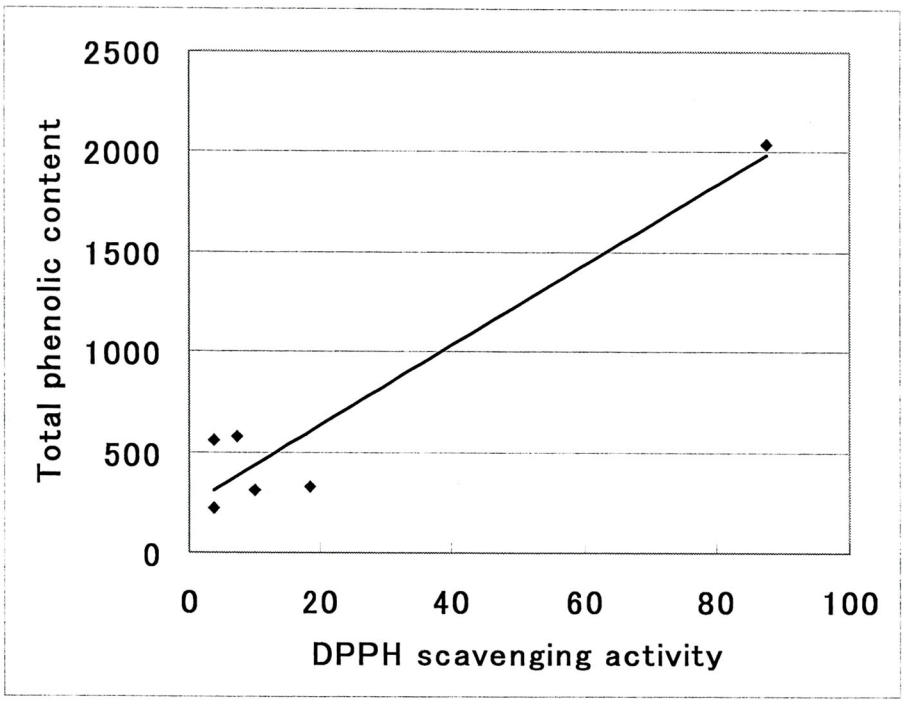
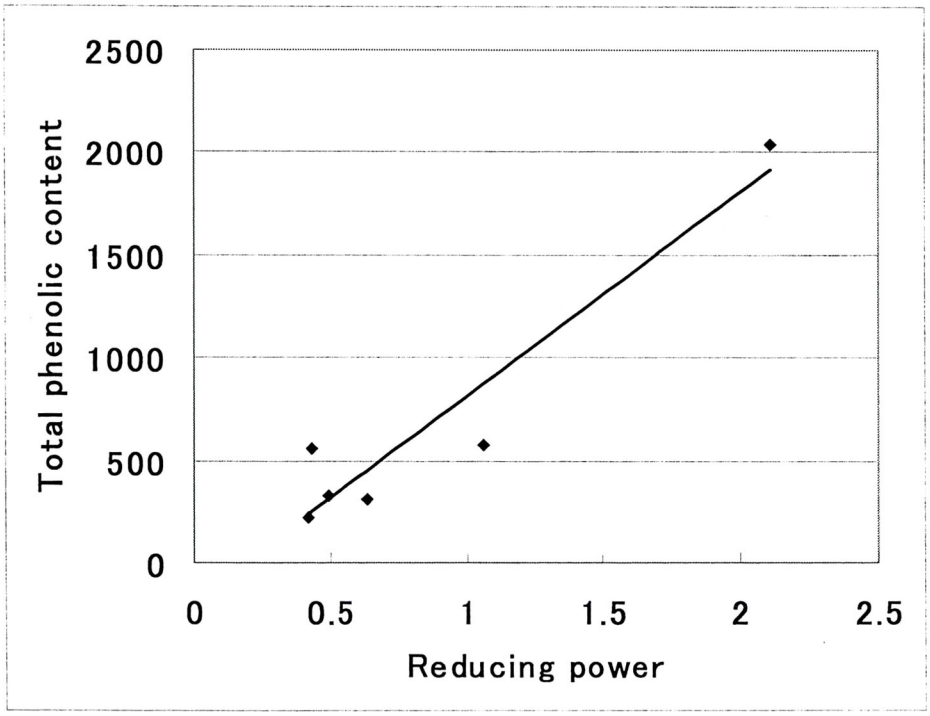


Figure 3.1. Dose-dependency of the DPPH radical scavenging activities of *S. caseolaris*. Data are mean ± SD (bars) values from three experiments.



a)



b)

Figure 3.2. a) Relationship between total polyphenol content and the DPPH radical scavenging activities of different extracts. The values for total polyphenol content are means ± SDs (bars) from three experiments.
b) Relationship between total polyphenol content and the reducing power of different extracts. The values for total polyphenol content are means ± SDs (bars) from three experiments.

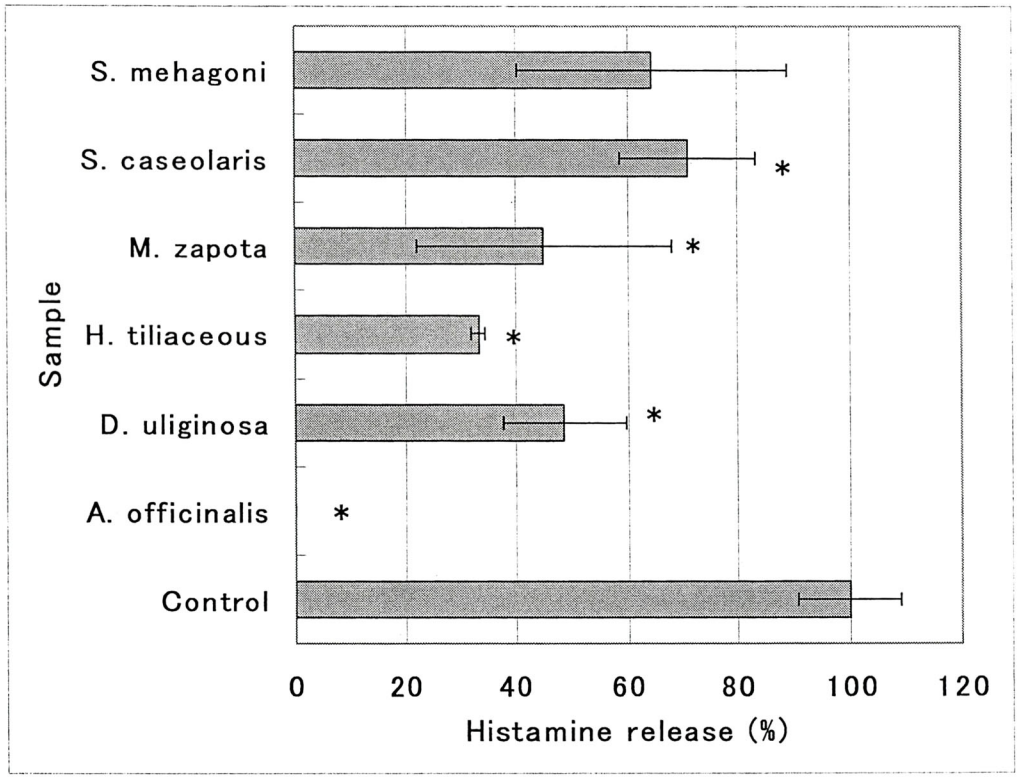


Figure 3.3. Inhibition of histamine release activity by the extracts of six Bangladeshi plants. The release of histamine from rat PECs caused by the Ca^{2+} ionophore A23187 was measured in the presence and absence of the extracts at 2 μ M GAE. The data are shown as means $\pm$ SD (bars) for four experiments. *$P < 0.05$ by Student's t test for the comparison between the control and extracts.

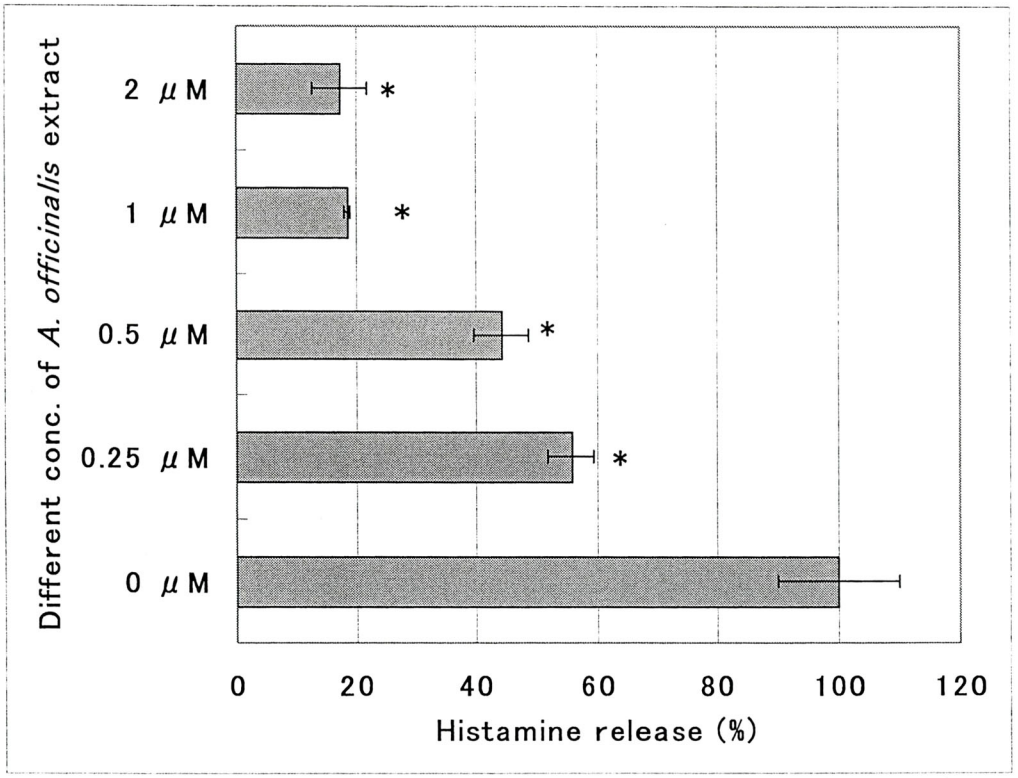


Figure 3.4. Dose-dependent inhibition of histamine release by the extract of *A. officinalis*.
The release of histamine from rat PECs caused by the Ca^{2+} ionophore A23187 was measured in the presence of various concentrations of the extract. The data are shown as means ± SD (bars) for four experiments. $*P < 0.05$ by Student's *t* test for the comparison between the control and extract.

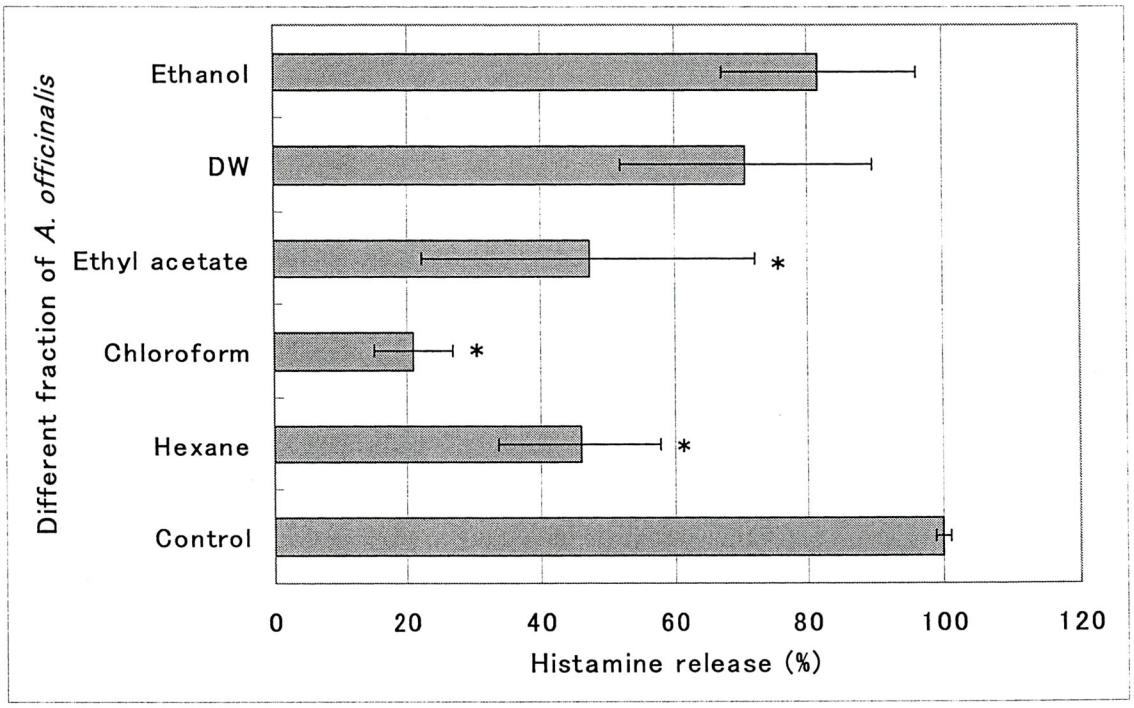


Figure 3.5. Inhibition of histamine release activity by the fractions (Hex, Chl, EtOAc, EtOH and DW) prepared from the leaves of *A. officinalis*. The release of histamine from rat PECs caused by the Ca^{2+} ionophore A23187 was measured in the presence and absence of the extracts at 2 μM GAE. The data are shown as means ±SD (bars) for four experiments. $*P < 0.05$ by Student's *t* test for the comparison between the control and extracts.

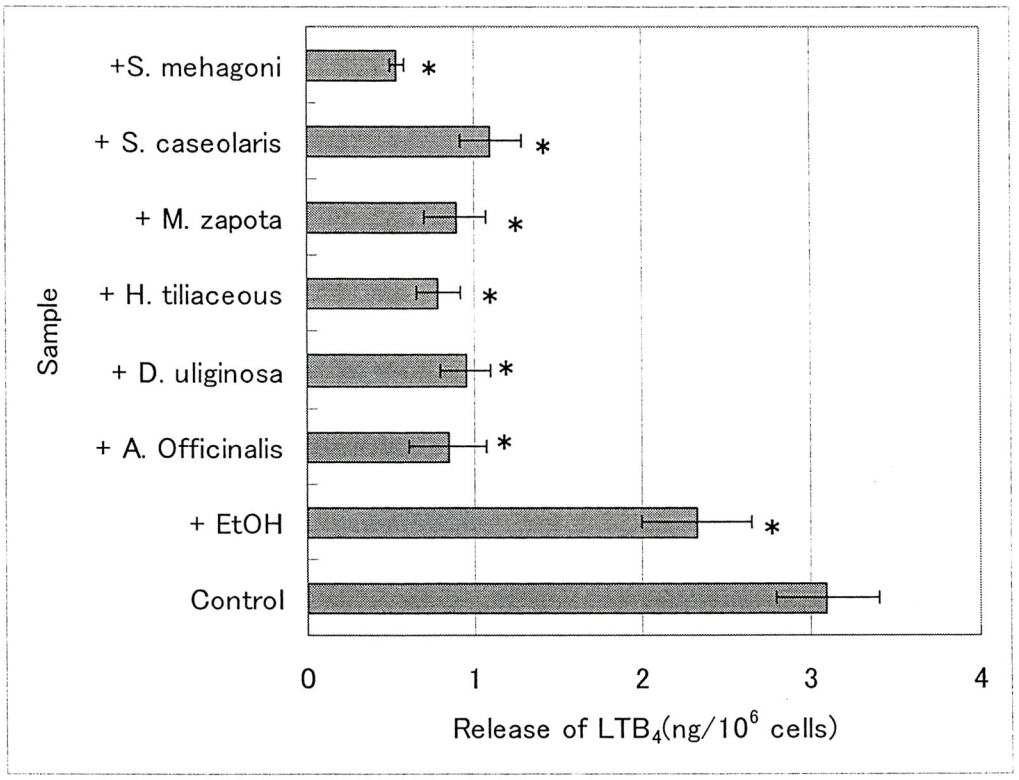


Figure 3.6. Inhibition of LTB_4 release activity by the extracts of six Bangladeshi plantsThe release of histamine from rat PECs caused by the Ca^{2+} ionophore A23187 was measured in the presence and absence of the extracts at 2 μM GAE. The data are shown as means ± SD (bars) for four experiments. *$P < 0.05$ by Student's *t* test for the comparison between the control and extracts.

Table 5: Analgesic effect of the ethanolic extract of *D. uliginosa* on acetic acid-induced nociception

Group	Dose (mg/kg)	No. of writhing (mean ± SD)	Inhibition (%)
Control	-	76.66 ± 1.53	-
Diclofenac sodium	25	17 ± 1	77.82
D. uliginosa	200	32.50 ±0.07	57.60
D. uliginosa	400	29.33 ± 2.08	61.74

Values are mean ± SD, n=3.

3. 2. Anti-histamine release and anti-inflammatory activity of aqueous extract of citrus fruit (*Citrus* sp.) peels.

INTRODUCTION

Citrus fruits are among the most popular in the world with more than 100 million tons produced each season. Moreover, large amounts of peel must be disposed of (Li *et al.*, 2006) since about one third of the yield of citrus fruits is used for the production of juice. The peel contains various beneficial bioactive components including ascorbic acid, carotene, limonene, polyphenols such as hesperidine, and fibers (Bocco *et al.*, 1998; Gorinstein *et al.*, 2001). With the aging of societies and increases in physical and mental stress, it is important to find beneficial activities from new botanical resources which are safe and economical, and can be used in supplements or drugs.

In early spring, allergies to cedar pollen are common in Japan. An allergy is a form of immune dysfunction and can be classified into four types. Type 1 plays an important role in the expression of allergies to foods and environmental allergens (Younginger, 1992; Marks and marks, 1993). Mast cells play a crucial role in the many physiological changes that occur during an immediate allergic response through the production and release of chemical mediators such as histamine and eicosanoid (Kaliner *et al.*, 1982; Matsuo *et al.*, 2000). So, compounds which inhibit histamine's release from mast cells will reduce the symptoms of allergies. Reportedly, various polyphenols in foods and beverages induce anti-allergic effects (Corvazier and Maclouf, 1985; Kanda *et al.*, 1998; Takasugi *et al.*, 2002; Park et al., 2008; Itoh *et al.*, 2008).

In a previous paper (Hossain *et al.*, 2008), antioxidative, anti-amylase, anti-glucosidase, and anti-histamine release activities were found in Bangladeshi fruits. Some EtOH extracts of Bangladeshi medicinal plants potentiated the response of $GABA_A$ receptors expressed in *Xenopus* oocytes and extended sleeping time in mice given by pentobarbital (Mubassara *et al.*, 2009). An aqueous extract of citrus peel reduced the production of hydrogen peroxide in catechin-enriched green tea (Ayabe and Aoshima, 2007). In this paper, both anti-histamine release and anti-inflammatory activities were investigated, using aqueous extracts of citrus peels. The aim of the study was to identify natural sources, which are economical and safe, and could be used to prepare functional foods, supplements, and drugs with anti-allergic activity.

RESULT

Measurements of the total phenolic content of aqueous extracts of the peels of orange, grapefruit, lime and lemon made by the Folin-Ciocalteu method are shown in **(Fig. 3.7)**. All extracts contained high concentrations of phenols, the order being orange > lemon> lime > grapefruit.

The inhibition of histamine's release from rat PECs (1×10^6/mL) by the aqueous extracts was examined, where the release was stimulated with 5 μM A23187 (calcium ionophore) for 20 min. As shown in **(Fig. 3.8)**, all extracts inhibited the release of histamine from the cells. The inhibition by the extracts heated at 100 °C for ten minutes was greater than that by un-heated extracts (Ho and Lin, 2008), though only the extract of grapefruit peel increased the inhibition significantly when it was heated at 100 °C for ten minutes. To examine the effect of preincubation with the extracts, PECs were cultured with the extracts of citrus peels for ten minutes and the release was stimulated by the addition of A23187. No significant increase in the inhibition of histamine

release from the PECs was observed when the PECs were preincubated with the extracts (data not shown).

Since grapefruits are easily obtained from local markets every season, they were used for further experiments. The dose-dependence of the inhibition of histamine's release by the extract of grapefruit heated at 100 °C for ten min was examined and is shown in **(Fig. 3.9)**. The extract inhibited the release dose-dependently.

To examine its anti-inflammatory activity, the aqueous extract of grapefruit peel was administered orally to mice. Both the heated and un-heated extract decreased the effusion of Chicago sky blue in the intraperitoneal cavity significantly compared with the control as shown in **(Fig. 3.10)**. When the extract heated at 100 °C for ten min was administered orally, effusion of the dye decreased to the level of that for the positive control, indometacin, an anti-inflammatory drug.

DISCUSSION

The peel of citrus fruits is reported to have several beneficial activities (Murakami *et al.*, 2000). In traditional Chinese medicine, *chen pi*, the dried peel of *Citrus reticulate* has been widely used for centuries as a remedy to treat indigestion and to improve inflammatory syndromes of the respiratory tract such as bronchitis and asthma. Previously, we reported that an aqueous extract of citrus peel reduced the production of hydrogen peroxide in catechin-enriched green tea (Ayabe and Aoshima, 2008).

Aqueous extracts of all citrus peels examined inhibited the release of histamine from PECs. Heating of the extracts increased the inhibitory activity. The active components in the peel are unlikely to be proteins. Ho and Lin (2008) measured the inhibitory effect of heat-treated citrus peel extracts upon NO production by lipopolysaccharide-activated RAW 264.7 macrophages and suggested that heat treatment helped to release nobiletin and tangeretin, which were possibly responsible for the increased anti-inflammatory activity.

Anti-allergic activities of polyphenols have been reported by various groups. Park *et al.* (2008) studied the effect of six flavonoids (astragalin, fisetin, kaempferol, myricetin, quercetin, and rutin) on mast cell-mediated allergic inflammation. They found that all except astragalin inhibited the release of histamine in RBL-2H3 cells and that fisetin, quercetin and rutin decreased the gene expression and production of proinflammatory cytokines. These flavonoids down-regulate mast cell activation. Itoh *et al.* (2008) reported that polymethoxy flavones in fruit tissues of *Citrus* species inhibited the degranulation of chemical mediators in rat basophilic leukemia RBL-2H3 cells. It was suggested that the suppression of antigen-stimulated degranulation by the polymethoxy flavones was due to the Syk/PLC γ s/PKC pathway and Ca^{2+} influx. Though endogenous reactive oxygen species are involved in the regulation of degranulation, intracellular reactive oxygen species are not associated with the inhibition of degranulation by polymethoxy flavones, since they did not suppress intracellular reactive oxygen species and exhibited radical-scavenging activity. In our experiment, preincubation of the PECs with the extracts did not further inhibit the release of histamine, suggesting that it takes a little time to suppress the release. So flavonoids in aqueous extracts of peels of four citruses examined may modulate signal transduction in mast cells faster than the influx of Ca^{2+} induced by the Ca^{2+} ionophore, A23187, though precisely how is not clear.

Tachibana *et al.* (2004) have cloned the gene of the receptor for epigallocatechin-3-O-gallate (67-KDa laminin receptor) and found that anti-allergic activities such as the inhibition of histamine's release and suppression of the high-affinity IgE receptor (FcepsiolonRI) expression induced by epigallocatechin-3-*O*-(3-*O*-methyl)-gallate were caused by its binding to the laminin receptor (Fujimura *et el.*, 2007; Fujimura *et el.*, 2008). Various flavonoids in citrus peel are reported to have similar activities to epigallocatechin-3-*O*-(3-*O*-methyl)-gallate (Park *et al.*, 2008). However, the receptors for these flavonoids have yet to be found. It is necessary to clarify the primary determinant of the anti-allergic effect of these flavonoids and how the activities are induced.

Importantly, the oral administration of the aqueous extract of grapefruit suppressed the effusion of Chicago sky blue through capillary vessels in the intraperitoneal cavity of mice. Heating of the extracts suppressed the dye's effusion from capillary vessels to the positive control level, i.e., with indometacin. It is necessary to clarify how the flavonoids in citrus peel are incorporated into blood and to develop flavonoid derivatives which are easily absorbed into the blood-stream in the intestines.

Citrus peels contain various antioxidants such as ascorbic acid, hesperidine, β-cryptoxanthine and limonene, as well as polyphenols including flavonoids and induce anti-allergic activity. They also suppress the production of hydrogen peroxide from polyphenols under aerobic conditions. Several epidemiological studies have suggested that the consumption of fruit and vegetables is associated with a reduced risk of cardiovascular disorders and cancers (Ames, 1983; Kris-Etherton *et al.*, 2002), and neurodegenerative diseases such as Parkinson's and Alzheimer's diseases (Di Matteo and Esposito, 2003), as well as positive effects on inflammation and aging (Ames *et el.*, 1993). Peels of various citrus fruits are useful for developing supplements or drugs which are safe, cheap and easy to obtain in large amounts.

Acknowledgments: I want to acknowledge Ms. Tsujiyama Iyo of Yamaguchi University for her guidance and help during this study.

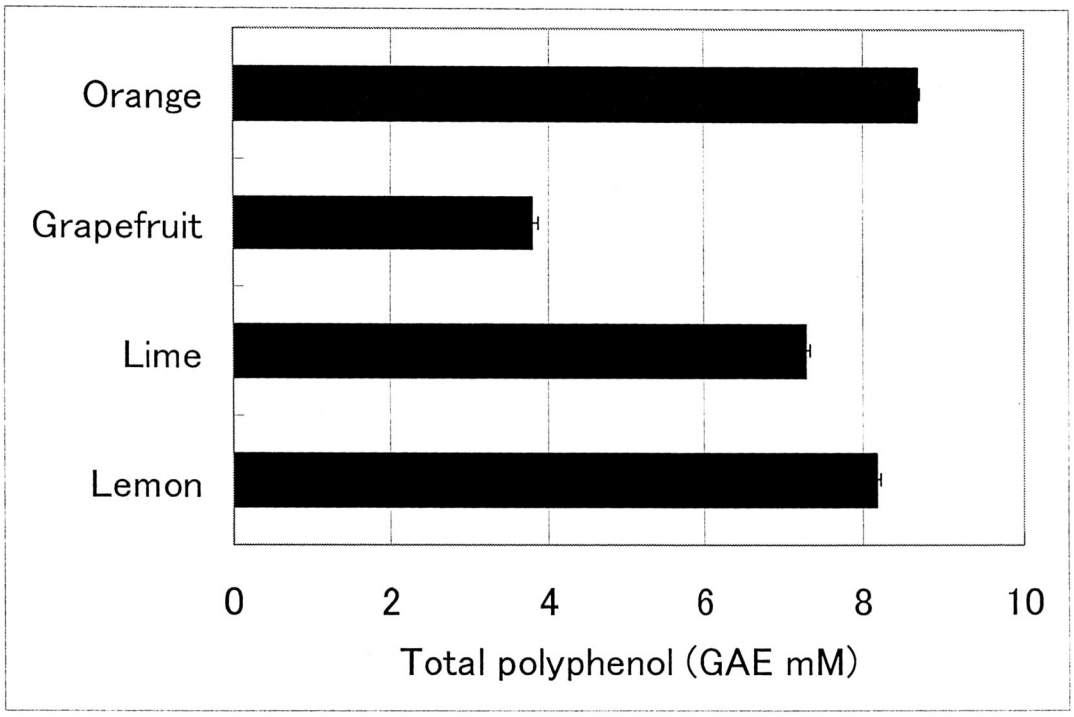


Figure 3.7. Total phenolic content of aqueous extracts of various citrus peels. Aqueous extracts of orange (*Citrus sinensis* Osbeck), grapefruit (*Citrus paradise* Mackfady.), lime (*Citrus aurantifolia* Christm.) and lemon (*Citrus limon* Burm.f) peel were prepared as described in Materials and Methods. The total phenolic content of the extracts was measured by Folin-Ciocalteu method with gallic acid (GA) as the standard and expressed (mM) as gallic acid equivalents (GAE)/mM of the extract. The data are shown as the mean ± SD (bars) for two experiments.

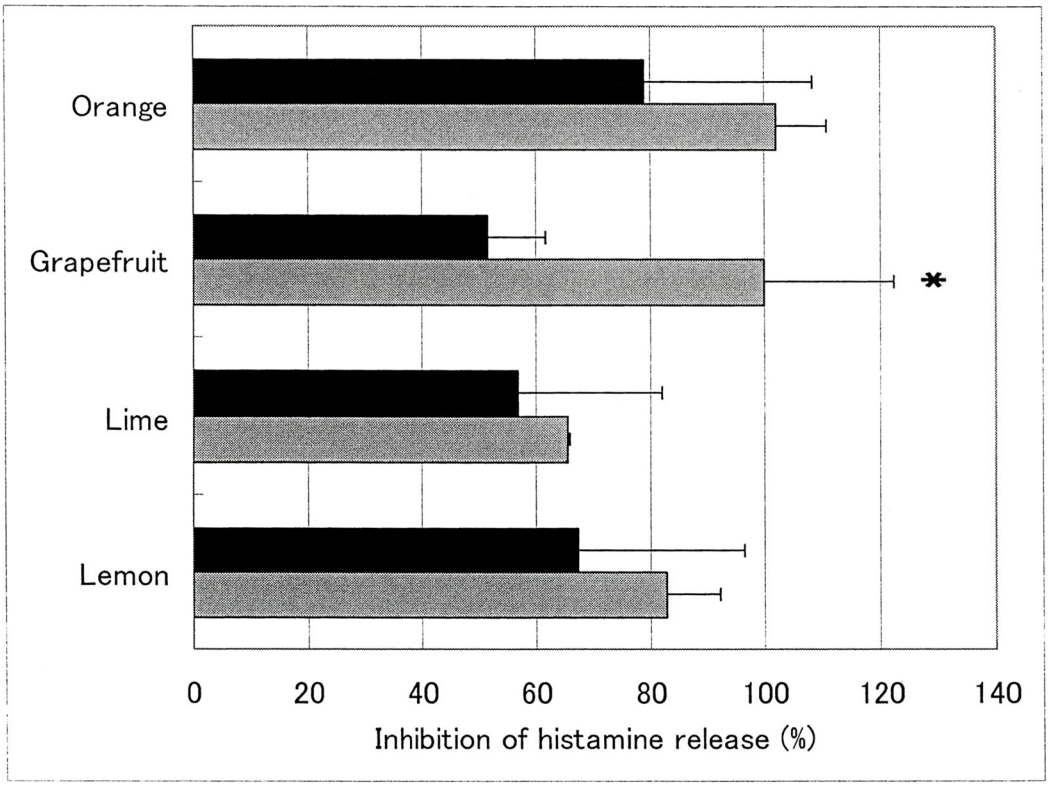


Figure 3.8. Inhibition of histamine-releasing activity by the aqueous extracts of citrus peels. The release of histamine from rat PECs caused by the Ca^{2+} ionophore A23187 was measured in the presence and absence of the extracts. Gray bars show the data when the extracts were heated at 100℃ for ten min, and black bars show the data for non-heated extracts. The data are shown as the mean ± SD (bars) for four experiments. *$P < 0.05$ by Student's t test for the values for heated and un-heated extract.

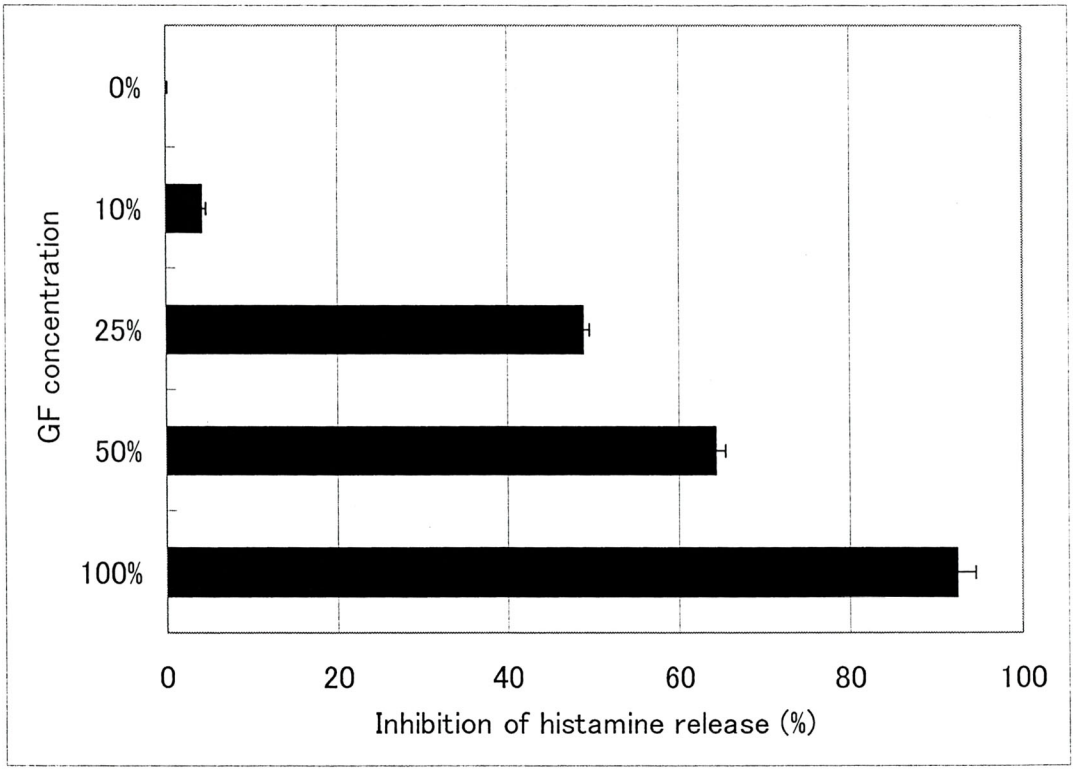


Figure 3.9. Dose-dependent inhibition of the release of histamine by the aqueous extract of grapefruit peel (GF) heated at 100℃ for ten min. The original extract of GF was diluted 10, 25, and 50 % with distilled water. The release of histamine from rat PECs caused by the Ca^{2+} ionophore A23187 was measured in the presence of various concentrations of the heated extract. The data are shown as the mean ± SD (bars) for three experiments. $P < 0.05$ by Student's t test for the values for the control and the extracts.

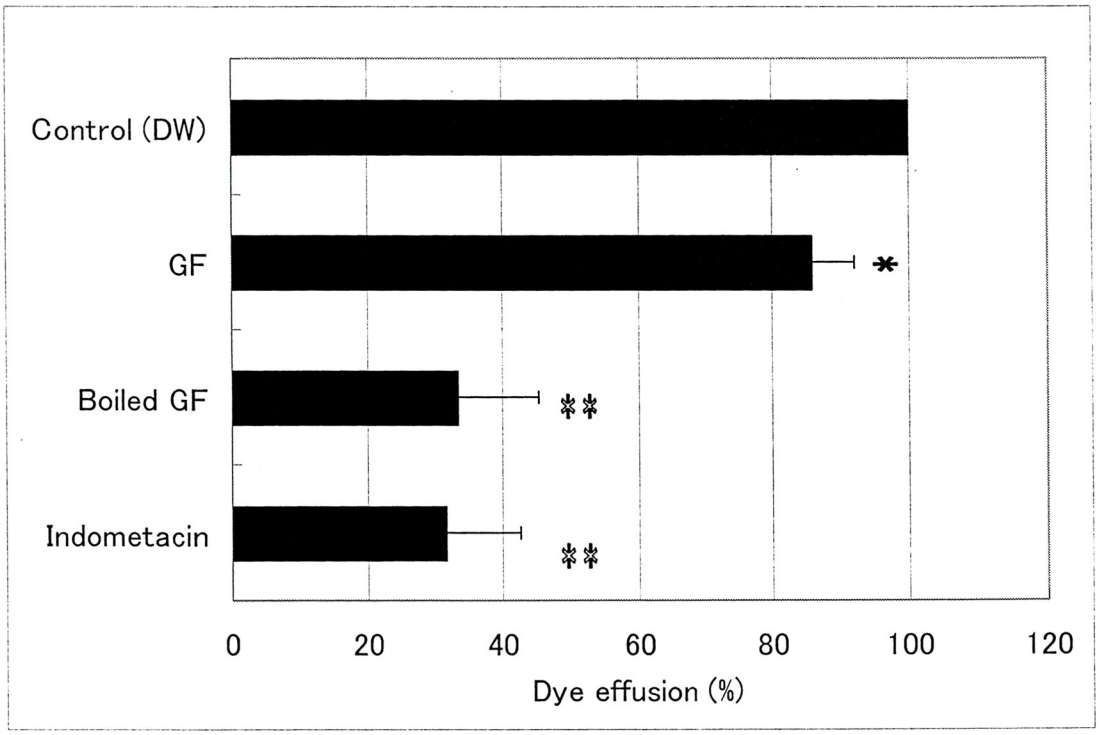


Figure 3.10. Suppression of the effusion of Chicago sky blue by the extracts of grapefruit peel. The heated or non-heated aqueous extract of grapefruit peel (GF) was administered orally to mice by an oral sonde. Distilled water was administered as a negative control, and indomethcin, as a positive control. After a venous injection of Chicago sky blue into mouse tail, 0.8% acetic acid dissolved in physiological saline was administered intraperitoneally to induce inflammation in the intraperitoneal cavity. After 10 min, the mice were killed by cervical dislocation and the intraperitoneal cavity was opened.to collect the dye which effused through capillary veins in the intraperitoneal cavity. The concentrations of Chicago sky blue were calculated from the absorbance at 590 nm. The data are shown as means $\pm$ SD (bars) for three experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ by Student's t test for the values for the negative control and the others.

CHAPTER FOUR

POTENTIATION OF RESPONSE OF $GABA_A$ RECEPTORS AND PENTOBARBITAL-INDUCED SLEEPING TIME IN MICE

4. 1. Effect of Bangladeshi medicinal plants on potentiation of the response of $GABA_A$ receptors and pentobarbital-induced sleeping time in mice.

INTRODUCTION

Various neurotransmitter receptors are involved in defining mental state, particularly the ionotropic γ-aminobutyric acid receptors ($GABA_A$ receptors), which are the main inhibitory neurotransmitter receptors in the human brain (Hossain *et al.*, 2007). These heteropentamers composed of various α, β, γ, δ and ε subunits were found to be expressed in *Xenopus laevis* oocytes (Trauner *et al.*, 2008). The potentiation of the response of these receptors by drugs such as benzodiazepine, pentobarbital and anesthetics induces tranquilizing, sleep-inducing or anesthetic responses in humans (Nicholls, 1994; Chebib and Johnston, 2000; Harrison *et al.*, 2000). It was found that fragrant compounds such as terpinen-4-ol, 1-octen-3-ol, and linalool potentiated the response of $GABA_A$ receptors expressed in *Xenopus* oocytes after injection of the receptor poly(A)$^+$RNA or cRNA (Aoshima and Hamamoto, 1999; Aoshima *et al.*, 2001; Aoshima *et al.*, 2006; Hossain *et al.*, 2002a, 2003, 2004, 2007; Hossain *et al.*, 2002b). Initially, $GABA_A$ receptors expressed in *Xenopus* oocytes injected with rat whole brain mRNA were used for measurements, but fragrances had similar effects on receptor response to that obtained on injecting cRNA of the α_1 and β_1 subunits of bovine $GABA_A$ receptors (Aoshima *et al.*, 2001). The potentiation site for alcohol, anesthetics and pentobarbital is present in $GABA_A$ receptors composed of only α and β subunits (Mihic *et al.*, 1997; Whiting *et al.*, 2000), though the γ subunit is necessary for potentiation of the $GABA_A$ receptor-mediated response by benzodiazepine (Gunther *et al.*, 1995). $GABA_A$ receptors composed of α_1 and β_1 subunits were used in the present study.

Fragrant compounds may modulate mood through potentiation of the $GABA_A$ receptor response after being absorbed into the brain because hydrophobic compounds are easily absorbed through the blood-brain barrier in the same way as tranquilizers, sleeping drugs, and anesthetics. $GABA_A$ receptor channels are modulated not only by clinically important drugs such as benzodiazepines, barbiturates and various general anesthetics, but also by several compounds of plant origin including flavonoids, such as methyl-apigenin (Sheghart, 1995) or eogonin (Hui *et al.*, 2002), polyacetylenes, (Baur *et al.*, 2005), monoterpenes, such as borneol (Granger *et al.*, 2005), and thymol (Garcia *et al.*, 2006). Perez *et al.* (1998) found neuropharmacological properties in the fruit of *Solanum nigrum* which possesses potential CNS-depressant action.

In this study, eleven Bangladeshi fruits and trees traditionally used as medicines were screened for potentiating effects on the response of the $GABA_A$ receptor together with prolonging effects on pentobarbital-induced sleeping time. The aim of this study was to find medicinal plants which can be used in the food and pharmaceutical industries for preparation of functional foods, drinks, supplements and drugs with tranquilizing and sleep-inducing activities.

RESULT

Potentiation of the response of $GABA_A$ receptors:

$GABA_A$ receptors were expressed in *Xenopus* oocytes by injecting cRNA of the α_1 and β_1 subunits of the bovine $GABA_A$ receptors as shown in **Fig. 1a, Fig. 2a and 3a**. Plant extracts (40 µg/mL) dissolved in frog Ringer solution induced no response when they were applied to the injected oocytes **(Fig. 4.1b, Fig. 4.2b, Fig. 4.2c and Fig. 4.3b)**, indicating the absence of GABA in the extracts. However, addition of 40 µg/mL of the extract of *S. caseolaris*, *T. arjuna*, *T. bellirica*, or *S. cumini* to the 10 µM GABA solution significantly potentiated the response of the $GABA_A$ receptors as shown in **Fig. 4.4**. The extracts of *D. indica*, *E. officinalis*, *H. tiliacious* and *T. chebula* tended to potentiate the response, while that of *D. uliginosa* showed slight inhibitory activity.

The dose-potentiation curves of the extracts of *S. caseolaris* and *T. bellirica* are shown in **Fig. 4.11a, b and c**. The dissociation constant (K_p) and maximum potentiation of the receptors (V_m) when all potentiation sites of the receptors were occupied by the compound were estimated to be 48 µg/mL and 191%, 15.5 µg/mL and 165.4% and 34.6 µg/mL and 197.1% respectively, with the assumption of a simple equilibrium between the compound and the receptor (Aoshima *et al.*, 2001). When the pentane extract of *S. caseolaris* was applied, potentiation of receptor response was observed in the pentane phase **(Fig. 4.1c)**, indicating that active components are lipophilic.

Pentobarbital-induced sleeping time in mice:

Pentobarbital induces sleep by potentiating the response of $GABA_A$ receptors (Nicholls, 1994). As compounds which potentiate the response have been shown to prolong sleeping time in mice given pentobarbital (Koda *et al.*, 2003; Hossain *et. al.*, 2007), we examined the effects of intraperitoneal administration of several extracts (100 mg/kg) on pentobarbital-induced sleep (50 mg/kg) **(Fig. 4.6a)**. **Fig. 4.6b** illustrates the close relationship (R-squared value: 0.922) observed between the extract-associated potentiation of $GABA_A$ receptor response **(Fig. 4.4)** and the extension of pentobarbital-induced sleeping time in mice **(Fig. 4.6a)**. The co-administration of the extracts of *S. caseolaris*, *T. bellirica, S. cumini* or *T. arjuna* with pentobarbital prolonged the sleeping time significantly. Oral administration of these extracts had similar effects on sleeping time, as shown in **Fig. 4.6c**. The dose-dependence of the effects on sleeping time of three extracts was measured as shown in **Fig. 4.7a, b and c**. These findings suggest that these extracts act on $GABA_A$ receptors and potentiate their response *in vivo* as well. The co-administration of the extract of *E. officinalis* or *T. chebula* with pentobarbital prolonged sleeping time slightly, while that of *D. uliginosa* did not prolong it at all (negative control) as shown in **Fig. 4.6a**.

Examination of the cytotoxicity of the samples:

To examine the cytotoxicity, *Xenopus* oocytes were incubated for 24 h at 17.5 °C in Barth solution containing 20 µg/mL or 50µg/mL of extract and their membrane potentials were measured electrophysiologically (**Fig. 4.8**). No significant reduction in membrane potential (about – 40 mV) was observed in the presence of any extract at 20 µg/mL. The addition of the *S. caseolaris* extract (20 µg/ml) increased the membrane potential in the minus direction. So this extract may have some components which promote Na^+/K^+ATPase activity in oocytes. However, a significant decrease in membrane potential was observed in the presence of the extract of *S. caseolaris* (-6.6 V) or *S. mahagaoni* (-17.8 V) at 50µg/mL, suggesting cytotoxicity at high concentrations.

DISCUSSION

$GABA_A$ receptors are major inhibitory neurotransmitter receptors in the brain responsible for various neurological states such as anxiety, wakefulness and seizures. Potentiation of the responses of $GABA_A$ receptors causes tranquilizing and sleep-inducing effects on the brain, like that of benzodiazepines or pentobarbitals. Therefore, natural products which potentiate the response of $GABA_A$ receptors could be used to reduce anxiety and mental disorders (Hossain *et al.*, 2007).

Recently in Japan, GABA and glycine have been used as food additives with claims that they will induce mental relaxation as they are agonists of major inhibitory neurotransmitter receptors in the brain. However, it is unlikely that GABA and glycine added to foods act on $GABA_A$ and glycine receptors in the central nervous system. This is because neurotransmitters, including GABA and glycine, are usually incorporated selectively into the brain by special transporters and do not pass through the blood-brain barrier freely because of their hydrophilicity. There is a possibility that GABA in the blood acts on metabotropic (G protein-coupled) GABA receptors ($GABA_B$ receptors) in the peripheral nervous system, inhibiting the release of noradrenaline from sympathetic nerves and decreasing blood pressure in hypertensive rats or humans (Hayakawa *et al.*, 2002). Conversely, hydrophobic compounds, such as fragrant compounds, will be incorporated into the brain and act on $GABA_A$ receptors in the central nervous system, as they pass through the blood brain barrier easily.

The addition of alcoholic extracts of *S. caseolaris, T. bellirica, S. cumini* and *T. arjuna*, which are medicinal fruits and trees used in Bangladesh, to a GABA solution potentiated the response of $GABA_A$ receptors expressed in *Xenopus* oocytes, though the extracts themselves induced no response. The methanol extracts of *A. officinalis, H. tiliaceous*, and *M. zapota* did not induce significant responses, possibly because these plants include few effective components. The pentane extract of *S. caseolaris* also potentiated the response, indicating that the active component(s) in the extract are hydrophobic. Moreover, the extracts of *S. caseolaris, T. bellirica, S. cumini* and *T. arjuna* prolonged pentobarbital-induced sleeping time additively, when administered to mice both intraperitoneally 30 min before the administration of pentobarbital and orally 5 h before the administration of pentobarbital. A close relationship (R-squared value= 0.922) was observed between the potentiation of the response of $GABA_A$ receptors **(Fig. 4.4)** and the extension of pentobarbital-induced sleeping time in mice given by the extracts **(Fig. 4.6a)**, suggesting that active components are incorporated into the brain and act on the $GABA_A$ receptors. The possibility cannot be excluded that the extracts inhibit the decomposition of pentobarbital in the liver and thus increase sleeping time in mice, but it is unlikely as these plants have been used as traditional medicines for a long time and no toxicity has been reported. These extracts have considerably less effect on $GABA_A$ receptors than synthetic drugs such as benzodiazepine and pentobarbital (Nicholls, 1994; Chebib and Johnston, 2000; Harrison *et al.*, 2000), but their side effects may also be much weaker. Since the extract of *S. caseolaris* at a high concentration, 50 μg/mL, was lethal to *Xenopus* oocytes, one needs to be careful about its cytotoxicity. However, the extract is less toxic than (-)-epigallocatechingallate or 3-*O*-octanoyl-(+)-catechin (Hossain *et al.*, 2002c; Aoshima *et al.*, 2005), which is a main component in tea, or a synthesized catechin derivative and induces cell death at concentrations above 10 μg/mL. Thus, when consumed, they will induce only slight mental relaxation and pose little risk. Therefore, these extracts may be of use for the development of supplements which improve mental health as tranquilizers by

potentiating the response of $GABA_A$ receptors.

S. caseolaris is a small tree found in tidal creeks and mangrove swamps in Bangladesh. The fruit is used as a poultice on sprains and swellings. The fermented juice of the fruit is useful in arresting hemorrhage and stop-bleeding treatment of piles (Kirtikar & Basu, 1987). The fruits of *T. bellirica* and *S. cumini* are used as medicinal treatments for hepatitis, coughing and hoarseness, and for anti-dysentery, inflammation, and diabetes mellitus, respectively. The bark of *T. arjuna* is used to treat hypotension, and as a cardiac tonic and febrifuge (Goni, 2003). Since these plants have long been used as medicinal products, they should be safe when used as supplements.

It remains necessary to identify the effective components of these active extracts. As $GABA_A$ receptors composed of α_1 and β_1 subunits were used for this study, a benzodiazepine-like compound is unlikely to be such a component (Gunther *et al.*, 1995).

Acknowledgments: I want to acknowledge Dr. Sheikh Julfikar Hossain and Ms. Shigemori, of Yamaguchi University for measurements of the responses of $GABA_A$ receptors of Bangladeshi medicinal plants.

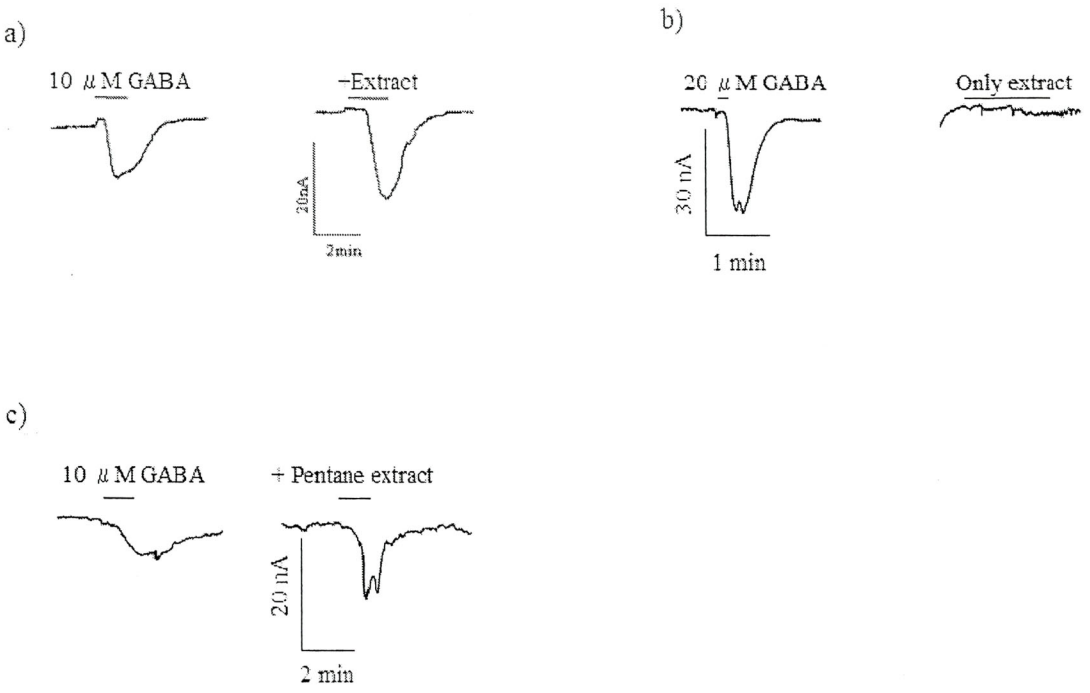


Figure 4.1. Effect of the extract of *S. caseolaris* on the response of *Xenopus* oocyte $GABA_A$ receptors, expressed by injection of receptor cRNA. Currents were measured with a voltage clamp at -40 mV. An inward current is shown as a downward curve. Two responses in a given panel were obtained from the same injected oocyte, but responses in panels a, b and c represent different oocytes.

a) Potentiation of $GABA_A$ receptor response by 40 μg/mL extract with 10 μM GABA. The upper bars indicate the timing of application of 10 μM GABA or the mixture of GABA and extract (40 μg/mL).

b) Receptor response induced by the ethanol extract of *S. caseolaris* only.

S. caseolaris (40 μg/mL) extract was applied to the oocyte expressing $GABA_A$ receptors. The response induced by 20 μM GABA was shown as a positive control.

c) Potentiation of $GABA_A$ receptor response by *S. caseolaris* in the pentane phase (40 μg/mL) in the presence of 10 μM GABA.

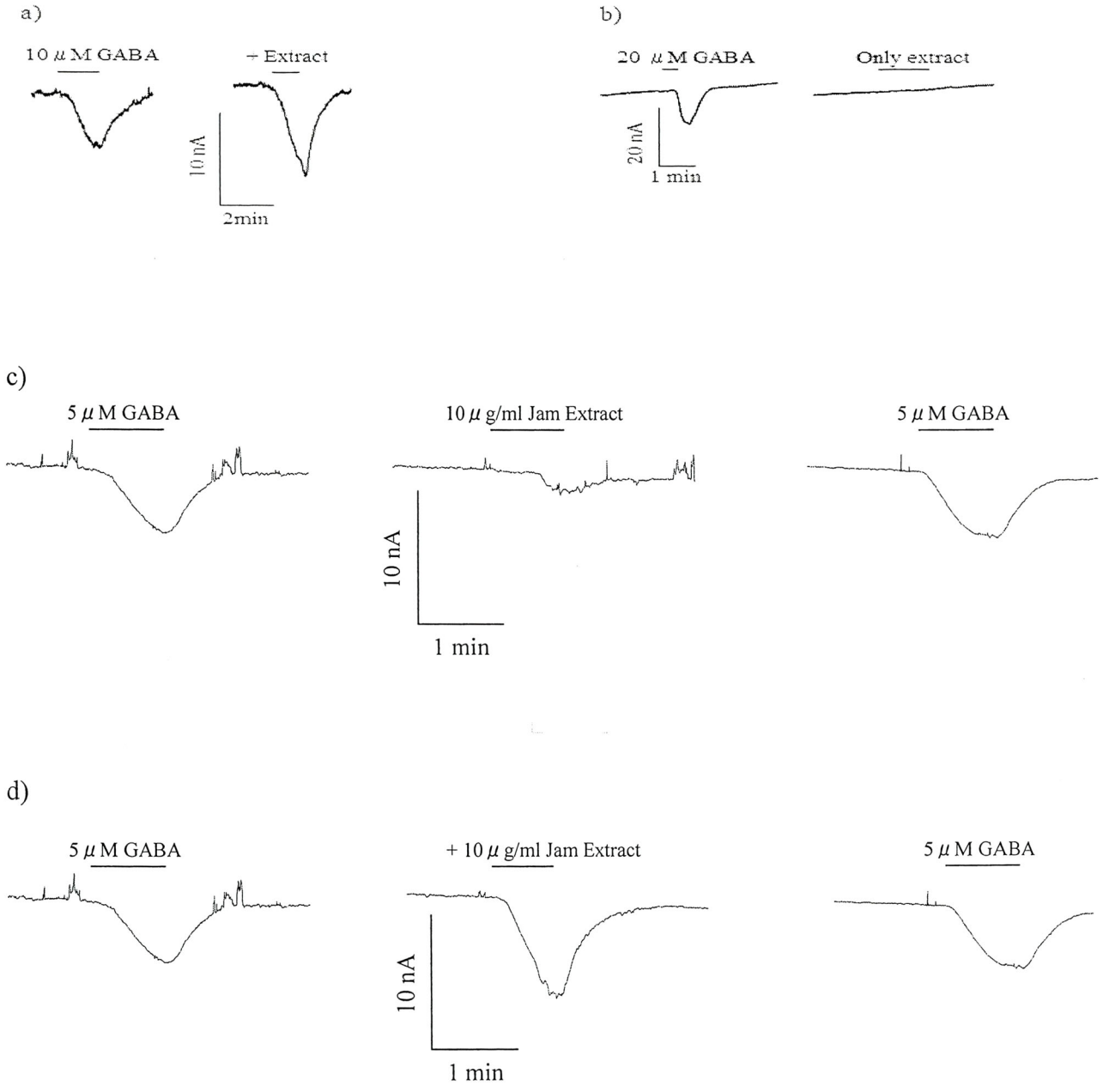
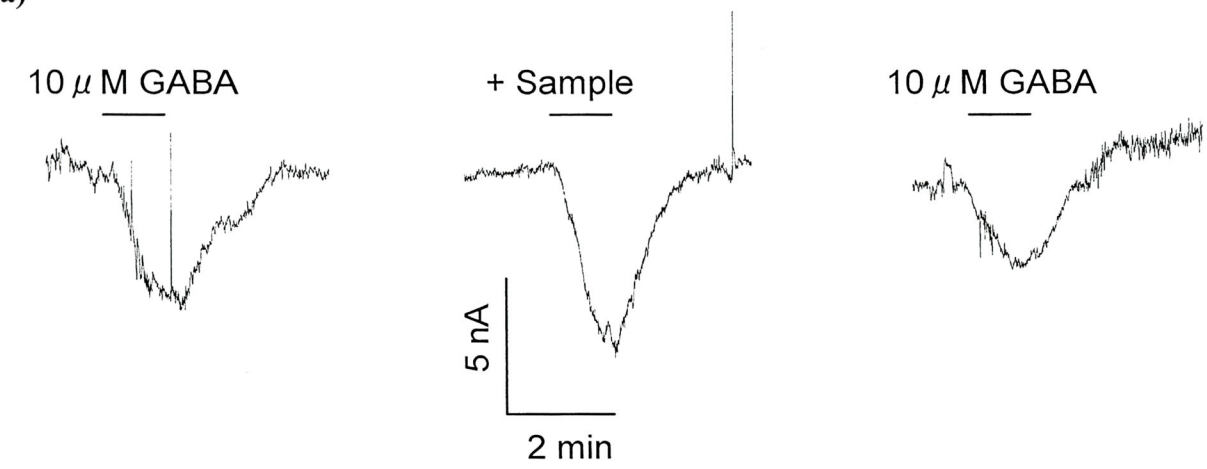


Figure 4.2. Effect of the extract of *T. bellirica* and *S. cumini* on the response of *Xenopus* oocytes $GABA_A$ receptors expressed by injection of receptor cRNA.

a) Potentiation of $GABA_A$ receptor response by ethanol extract of *T. bellirica* (40 μg/mL) in the presence of 10 μM GABA.
b) Receptor response induced by the ethanol extract of *T. bellirica* (40 μg/mL) only.
c) Receptor response induced by the ethanol extract of *S. cumini* (40 μg/mL) only.
d) Potentiation of $GABA_A$ receptor response by ethanol extract of *S. cumini* (40 μg/mL) in the presence of 10 μM GABA.

a)



b)

Figure 4.3. Effect of the extract of *T. arjuna* on the response of *Xenopus* oocytes $GABA_A$ receptors expressed by injection of receptor cRNA.

a) Potentiation of $GABA_A$ receptor response by ethanol extract of *T. arjuna* (40 μg/mL) in the presence of 10 μM GABA.

b) Receptor response induced by the ethanol extract of *T. arjuna* (40 μg/mL) only.

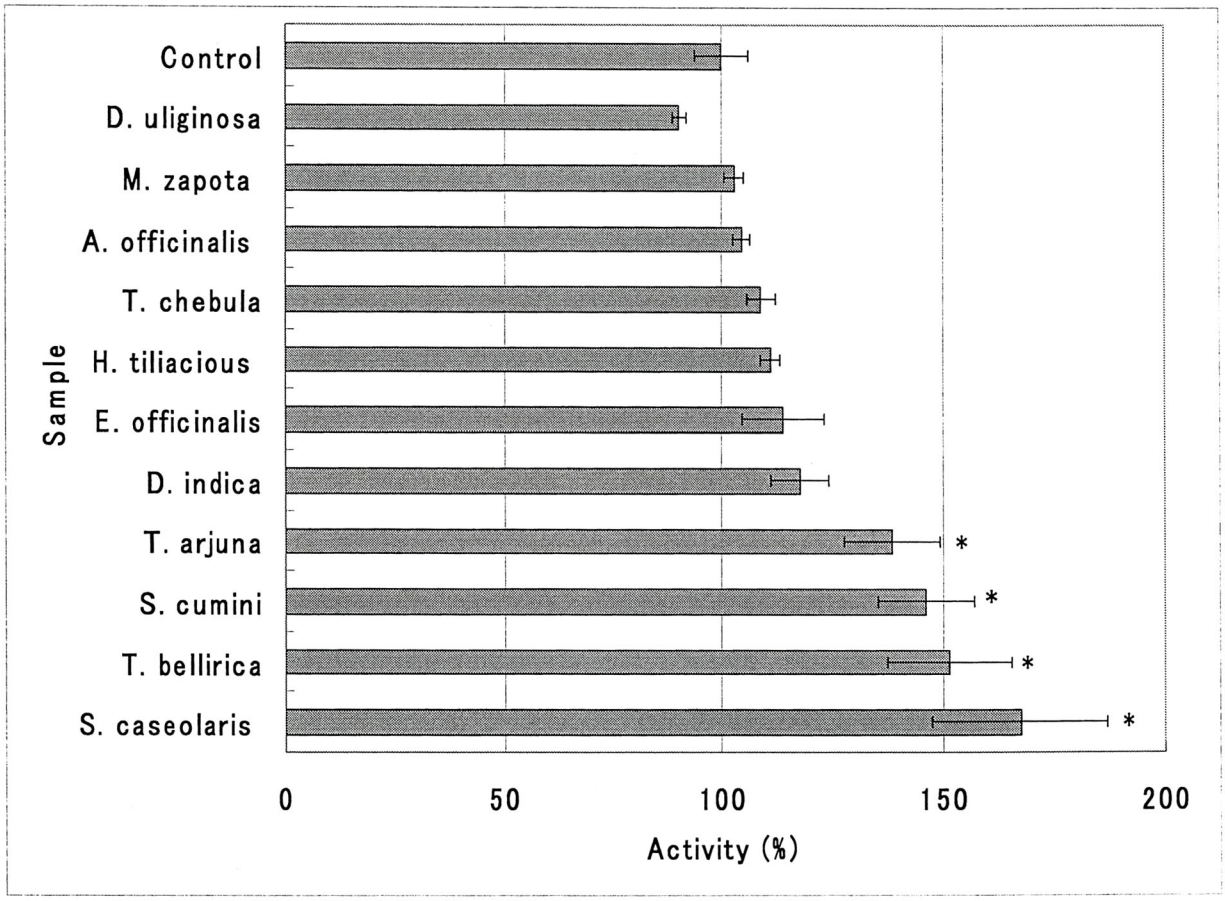
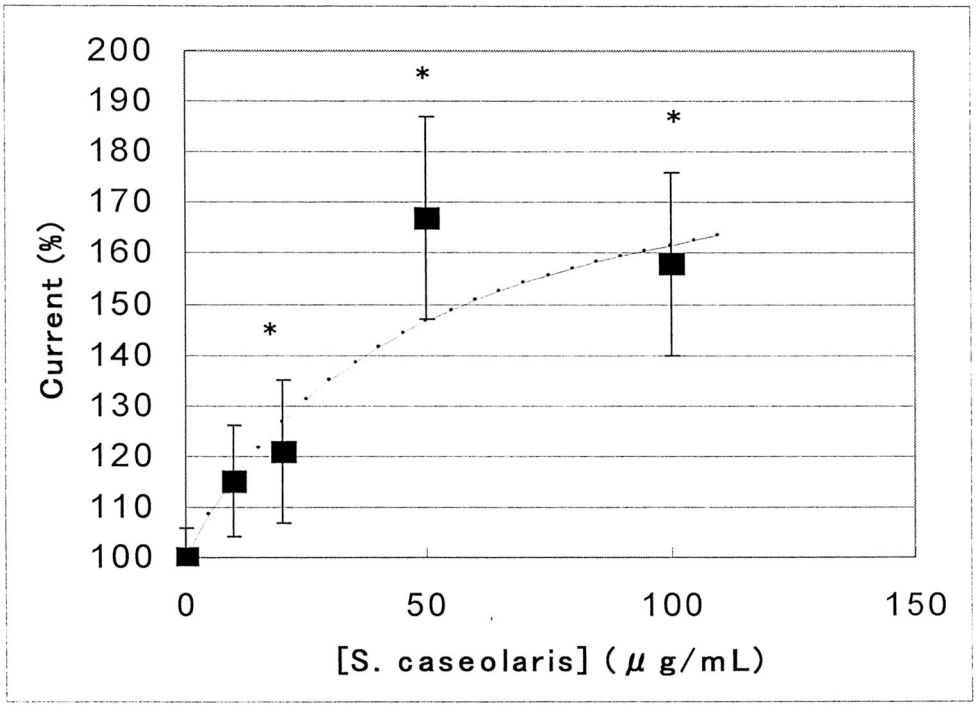
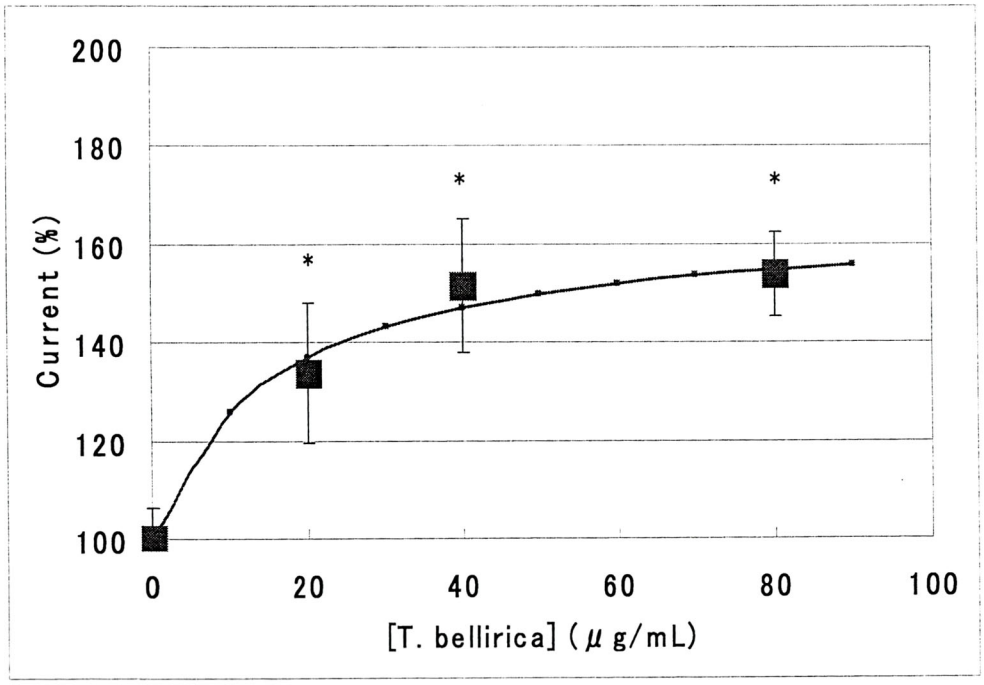


Figure 4.4. Effect of various plant extracts (40 μg/mL) on the 10 μM GABA-induced potentiation of $GABA_A$ receptor response. $GABA_A$ receptors were expressed in *Xenopus* oocytes by injecting cRNA prepared from cDNA for the α_1 and β_1 subunits of bovine $GABA_A$ receptors. The control response was obtained by perfusing 10 μM GABA solution without extract and was taken as 100%. Data are the mean ± SD (bars) of four experiments. $^*P<0.05$, Student's *t*-test of the mean values of the sample and those of the control.

a)


200
190
180
170
160
150
140
130
120
110
100
Current (%)
*
*
*
0
50
100
150
[S. caseolaris] (μ g/mL)

b)


200
180
160
140
120
100
Current (%)
*
*
*
0
20
40
60
80
100
[T. bellirica] (μ g/mL)

c)

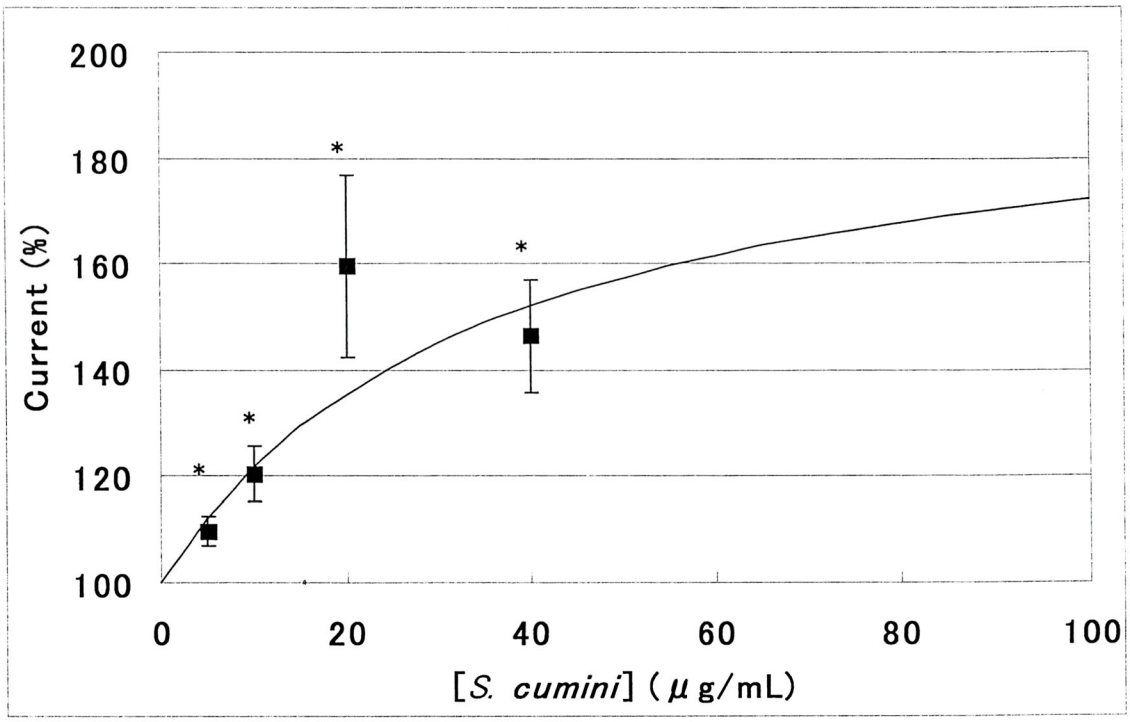
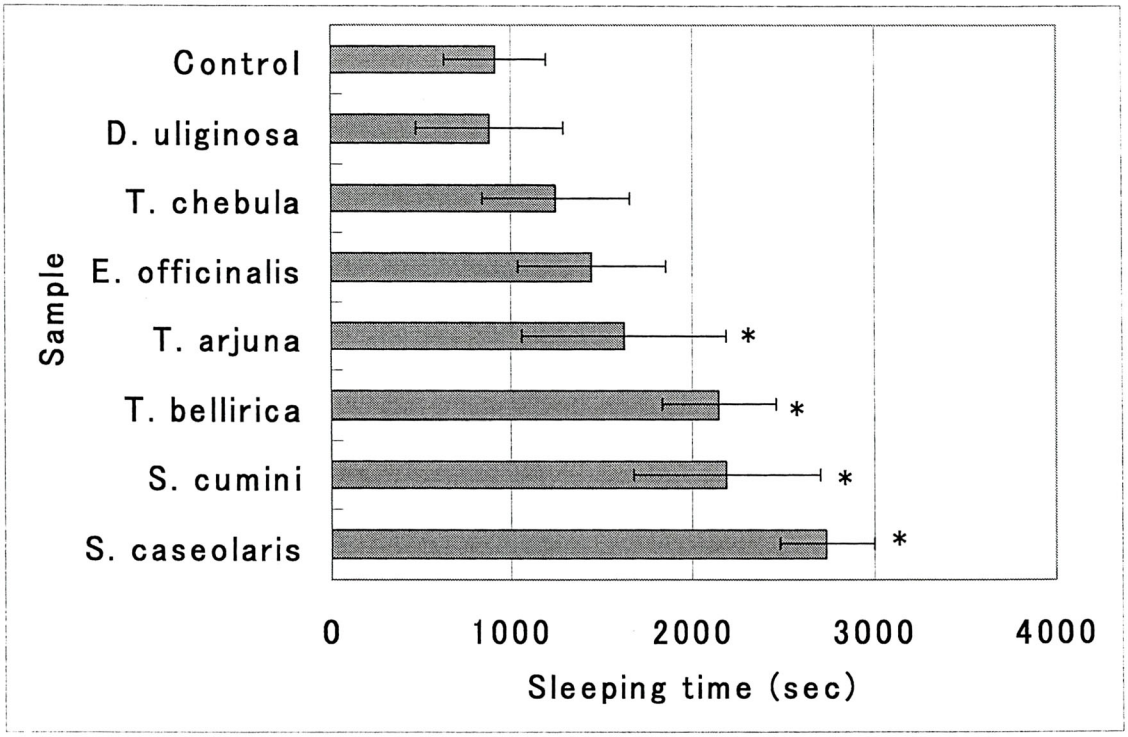
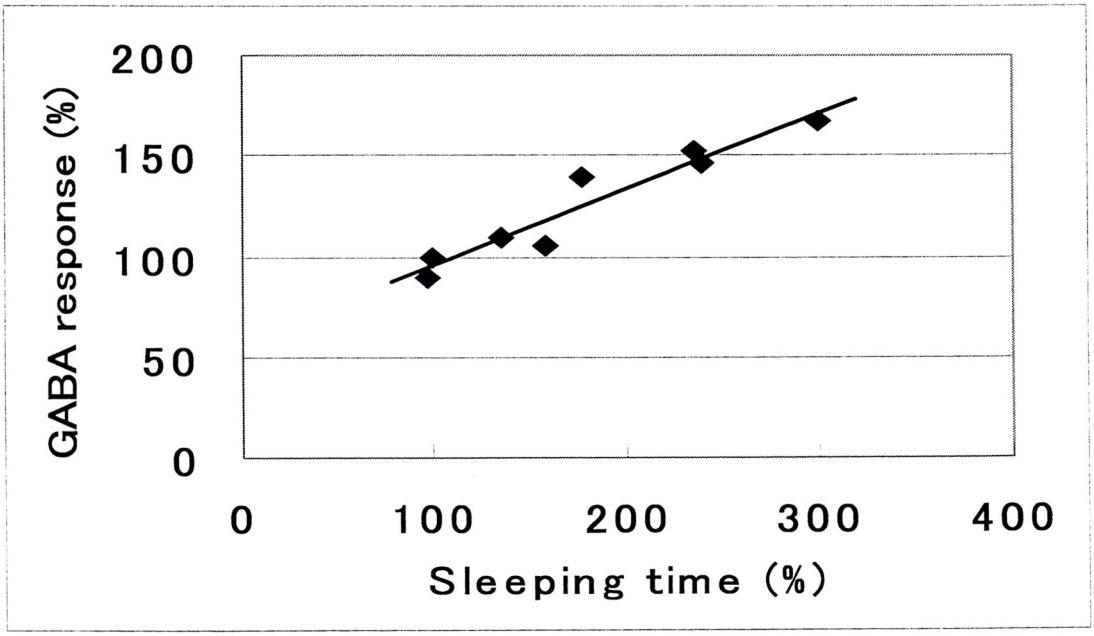


Figure 4.5. Dose-potentiation of the extracts of *S. caseolaris* (**a**), *T. bellirica* (**b**) and S. cumini (c). The extracts at various concentrations were applied simultaneously with 10 μM GABA. The control response was obtained by perfusing the GABA solution without extract and was taken as 100%. The theoretical curve was drawn using the values, K_p = 48 μg/mL and V_m = 191% (*S. caseolaris*), K_p = 15.5 μg/mL and V_m = 165.36% (*T. bellirica*) and K_p = 34.57 μg/mL and V_m = 197.08% (*S. cumini*) on the basis of a simple model (Aoshima *et al.*, 2001). The data are shown as mean ± SD (bars) of four experiments. *P<0.05, Student's *t*-test for comparison between the mean values of the sample and those of the control.

a)


Control
D. uliginosa
T. chebula
E. officinalis
T. arjuna
T. bellirica
S. cumini
S. caseolaris
Sample
*
*
*
*
0
1000
2000
3000
4000
Sleeping time (sec)

b)


200
150
100
50
0
GABA response (%)
0
100
200
300
400
Sleeping time (%)

c)

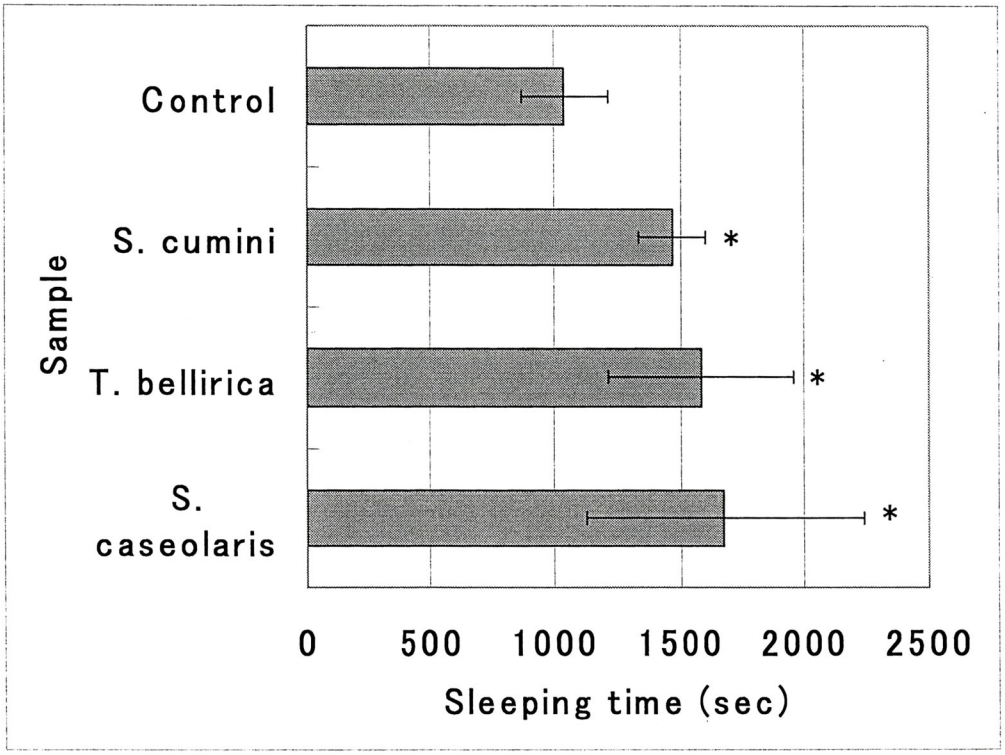
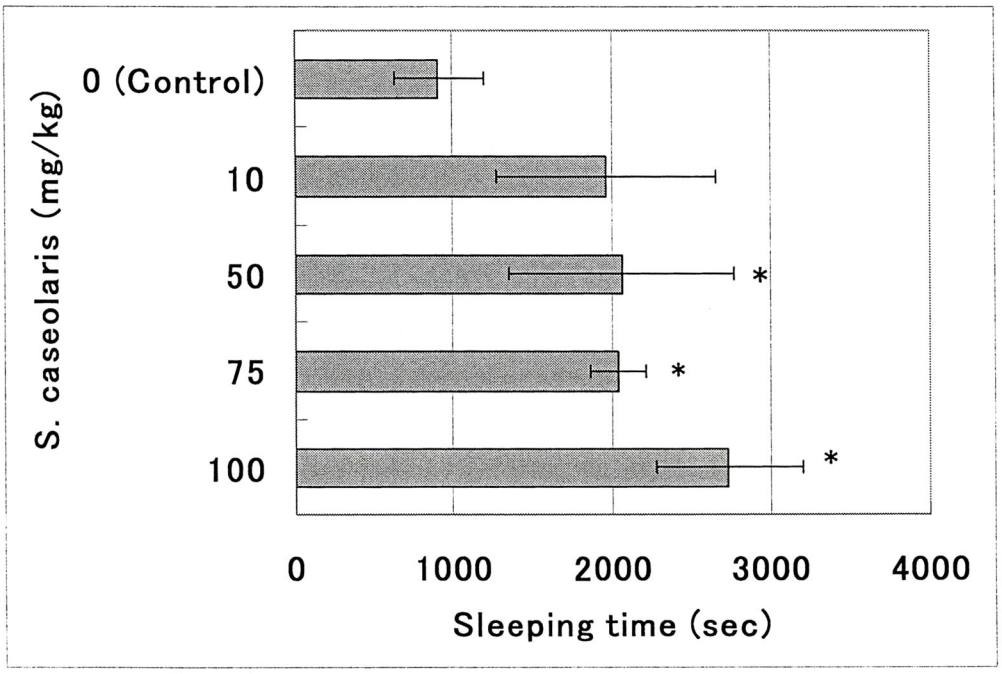
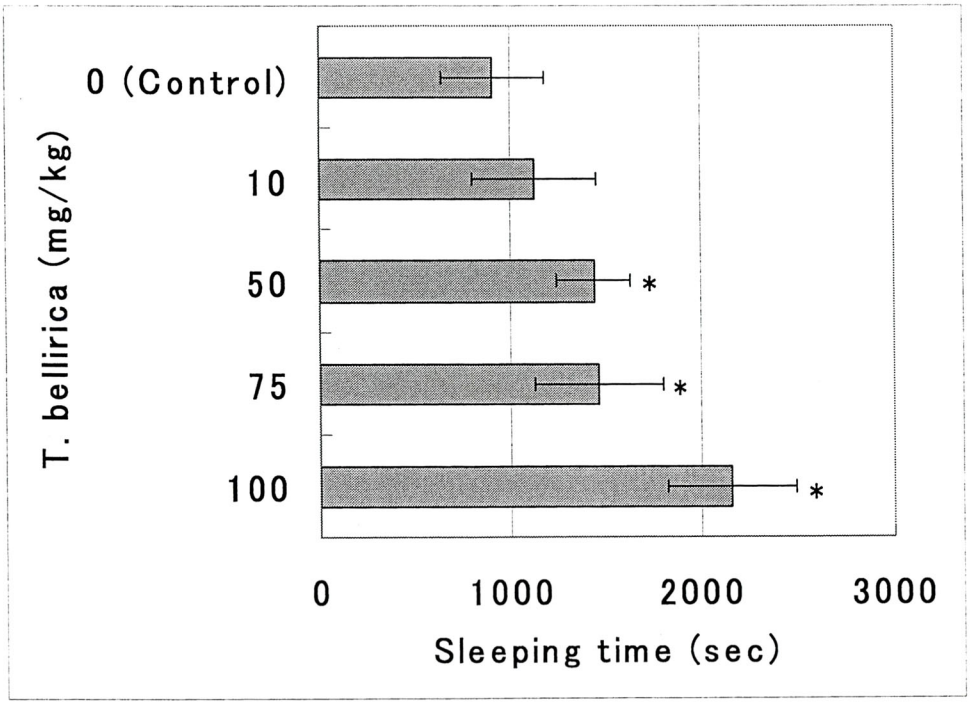


Figure 4.6. a) Effect of extracts on pentobarbital-induced sleeping time in mice. Pentobarbital (50 mg/kg) was injected into mice intraperitoneally 30 min after intraperitoneal injection of the extracts (100 mg/kg). Sleeping time was calculated as the time between disappearance and recovery of the righting reflex. The data are shown as mean ± SD (bars) for five experiments. The extract of *D. uliginosa* was used as a negative control. *$P<0.05$, Student's *t*-test for comparison between the mean values of the sample and those of the control.
b) Relationship between the potentiation of the response of $GABA_A$ receptors and the extract-associated extension of pentobarbital-induced sleeping time in mice. R-squared value of this relationship was calculated to be 0.922.
c) Effect of the oral administration of extracts on pentobarbital-induced sleeping time in mice. The mean oral administration doses of *S. caseolaris*, *S. cumini* and *T. bellirica* extract were 5.8 mg, 5.4 mg and 6.8 mg per mouse, respectively. As the average mouse weight was approximately 20 g, this represents administration of approximately 300 mg/kg of extract over 5 h.

a)


S. caseolaris (mg/kg)
0 (Control)
10
50
75
100
*
*
*
0
1000
2000
3000
4000
Sleeping time (sec)

b)


T. bellirica (mg/kg)
0 (Control)
10
50
75
100
*
*
*
0
1000
2000
3000
Sleeping time (sec)

c)

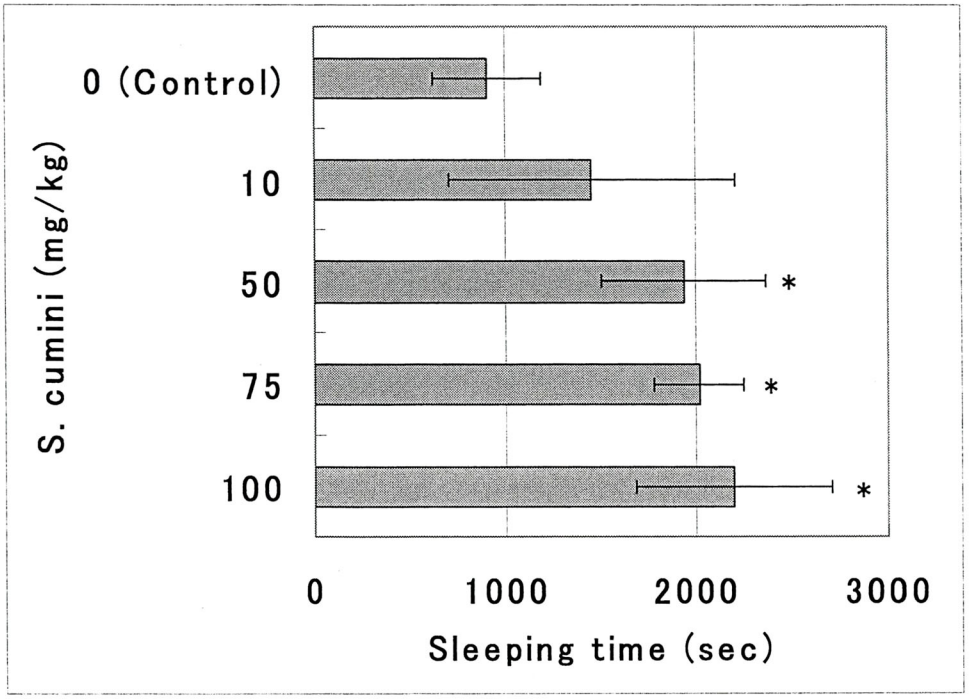


Figure 4.7. Dose-dependent effects of *S. caseolaris* (**a**), *T. bellirica* (**b**) and *S. cumini* (**c**) extracts on pentobarbital-induced sleeping time in mice. Pentobarbital (50 mg/kg) was injected into mice intraperitoneally 30 min after intraperitoneal injection of the extracts (10 - 100 mg/kg). *$P<0.05$, Student's *t*-test for comparison between the mean values of the sample and those of the control.

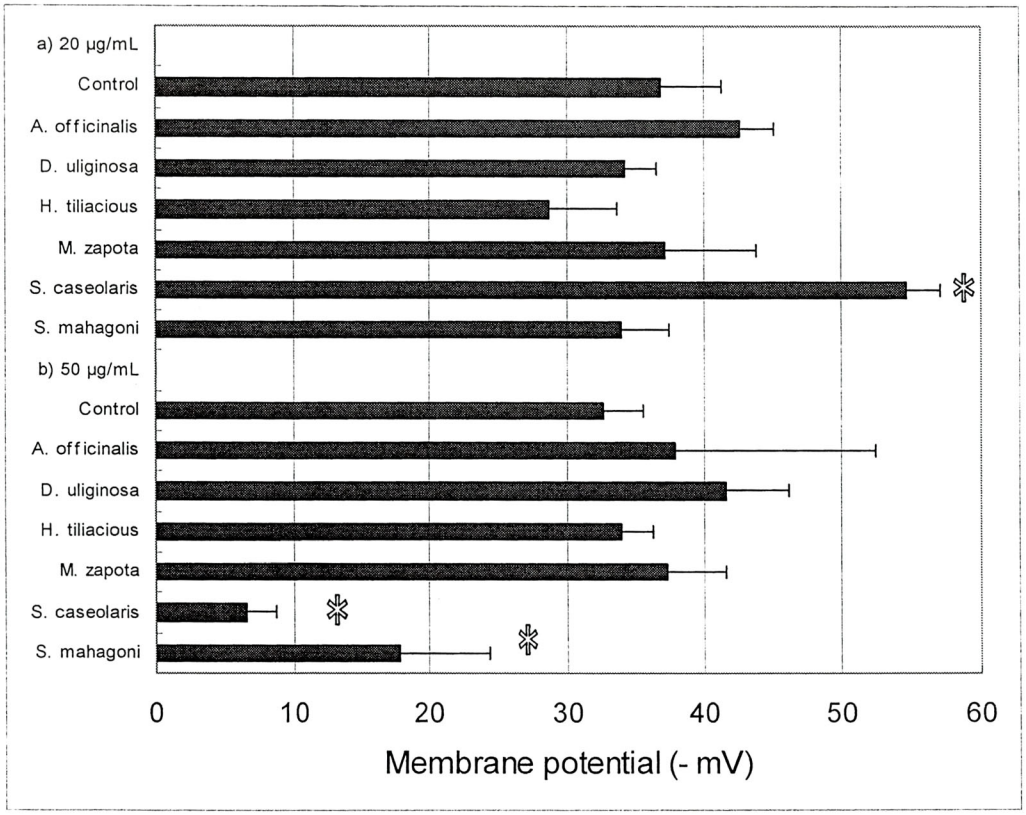


Figure 4.8. Effect of extracts of Bangladeshi plants on the membrane potential of *Xenopis* oocytes. The oocytes were incubated with six extracts at a final concentration of a) 20 mg/L or b) 50 mg/L for 24 h at 17.5℃. The membrane potential of the oocytes without the extracts were also measured as a control. Experiments a) and b) were conducted separately. The membrane potential of the oocytes was measured by inserting a microelectrode filled with 3 M KCl using a voltage clamp amplifier. The data are shown as means ± SD (bars) for five experiments. $*P < 0.05$ by Student's *t* test for the comparison between the control and extracts.

4.2. Effect of essential oils on the response of $GABA_A$ receptors and sleeping time in the mice induced by a sleeping drug.

INTRODUCTION

The $GABA_A$ receptor is a main inhibitory neurotransmitter receptor in the brain. The potentiation of the response of this receptor by drugs such as benzodiazepine, barbiturate, or anesthetics induces tranquillizing, sleep-inducing or anesthetic effects on human. As the drugs do, many fragrances in essential oils and beverages potentiated the responses of the $GABA_A$ receptors expressed in *Xenopus* oocytes by injection of their cRNAs. Co-administration of these fragrances to mice extended their sleeping time caused by pentobarbital, a sleep-inducing drug. These effects of fragrances may contribute in part to the sedative activity of essential oils in aromatherapy.

Essential oils or fragrances, which are used in aromatherapy, induce tranquility in the mind that is thought to come from stimulation of the olfactory system. Ultimately, this stimulates the limbic system, the hippocampus or the hypothalamus, the pituitary gland and the autonomic nervous system, the endocrine system or the immune system (Hayashi, 1998). Though the targets of fragrances have not been identified, it has also been proposed that fragrances have direct effects on the brain and modulate consciousness. It has been found that many fragrant compounds in essential oils and beverages potentiate the response of the $GABA_A$ receptors expressed in *Xenopus* oocytes and that they also increase the sleeping time induced by pentobarbital, which acts on the $GABA_A$ receptors. Here we tried to examine the effect of some essential oils and fragrant compounds on the response of the $GABA_A$ receptors as well as sleeping time experiment.

RESULT AND DISCUSSION

Potentiation of the responses of $GABA_A$ receptors by essential oils from cinnamon, coriander, clove and blended ones:

Essential oils from cinnamon, coriander, clove, and blended ones potentiated the response of the $GABA_A$ receptors caused by GABA solution **(Fig. 4.9 and Fig. 4.10). Fig. 4.9** shows example of electrical responses of the response of $GABA_A$ receptors potentiated by the essential oils. Their effects on the pentobarbital induced sleeping time of mice were examined by both intraperitoneal and inhalational administration prior to intraperitoneal administration of pentobarbital, which is known to act on $GABA_A$ receptors. Co-administration of essential oils prolonged the sleeping time. These essential oils may modulate our mood through acting on $GABA_A$ receptors and induce relaxed feeling while inhalation the oils.

Measurement of extension of pentobarbital-induced sleeping time of mice caused by essential oil and fragrance compound:

Essential oils from leaves of *Abies sachalinensis* Masters, wood of *Cryptomenia japonica* used as a barrel of sake storage and some fragrant compounds such as pinol, borniol and epi-cubinol were dissolved in olive oil and administered to mice intraperitoneally (200 mg/kg, 100 mg/kg, and 50 mg/kg). After 30 min. Pentobarbital was administered to the mice intraperitoneally (30 mg/kg). Then sleeping period was measured as the time between disappearance and recovery of the righting reflex. The results indicated that all the samples significantly increase the sleeping time induced by

pentobarbital in a dose dependent manner. Thus these essential oil and fragrant compounds have tranquillizing effects on the brain *in vivo*.

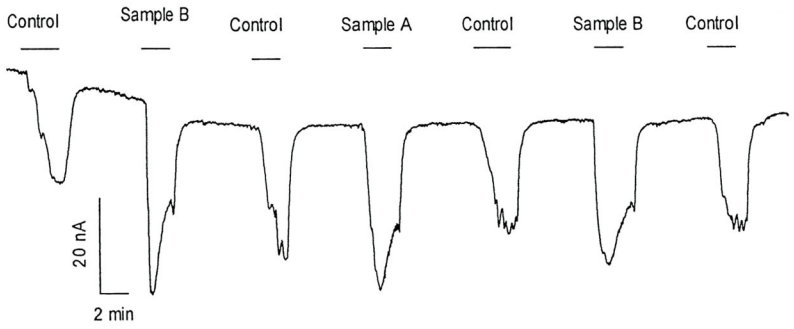


Figure 4.9. Effects of the essential oils on the GABA-elicited currents. All traces were obtained with a voltage clamp at -40 mV. An inward current is shown as a downward curve. The upper bars show when GABA or the mixture of GABA and the compound was applied. Both responses in a given panel were obtained from the same injected oocyte.

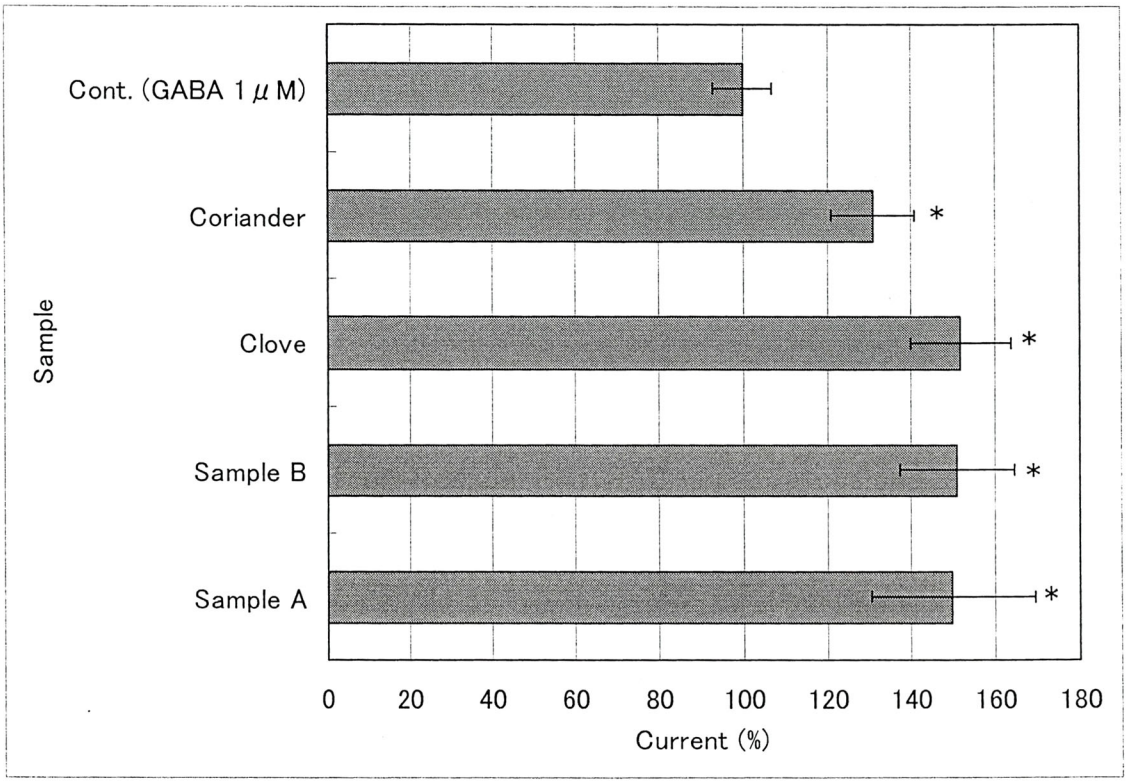


Figure 4.10. Potentiation of the $GABA_A$ receptors response by various essential oils. The response elicited by 1 μM GABA was taken as a control (100%).

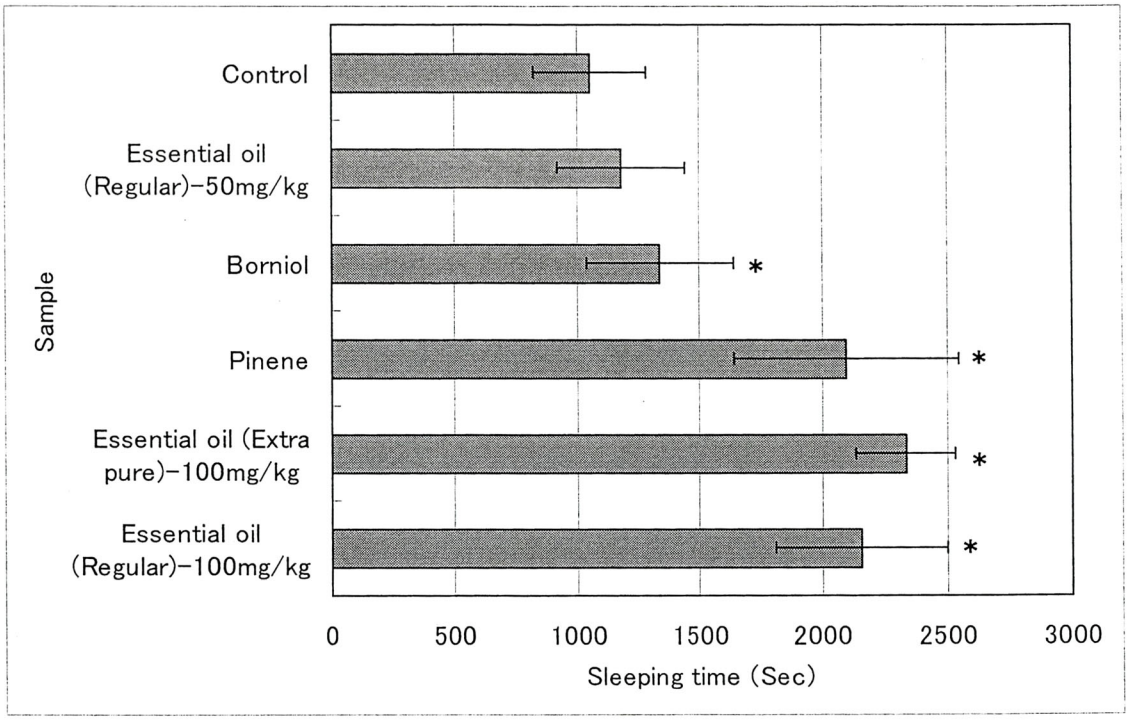


Figure 4.11. Effect of essential oil of *Abies sachalinensis* and fragrance compounds on the sleeping time induced by pentobarbital in mice. Sodium pentobarbital (30 mg/kg) was injected intraperitoneally 30 min after the administration of samples. Each value is the mean ± SD (bar) from four experiments. *$p < 0.05$ between the control value and the value in the presence of the component by Student's *t*-test.

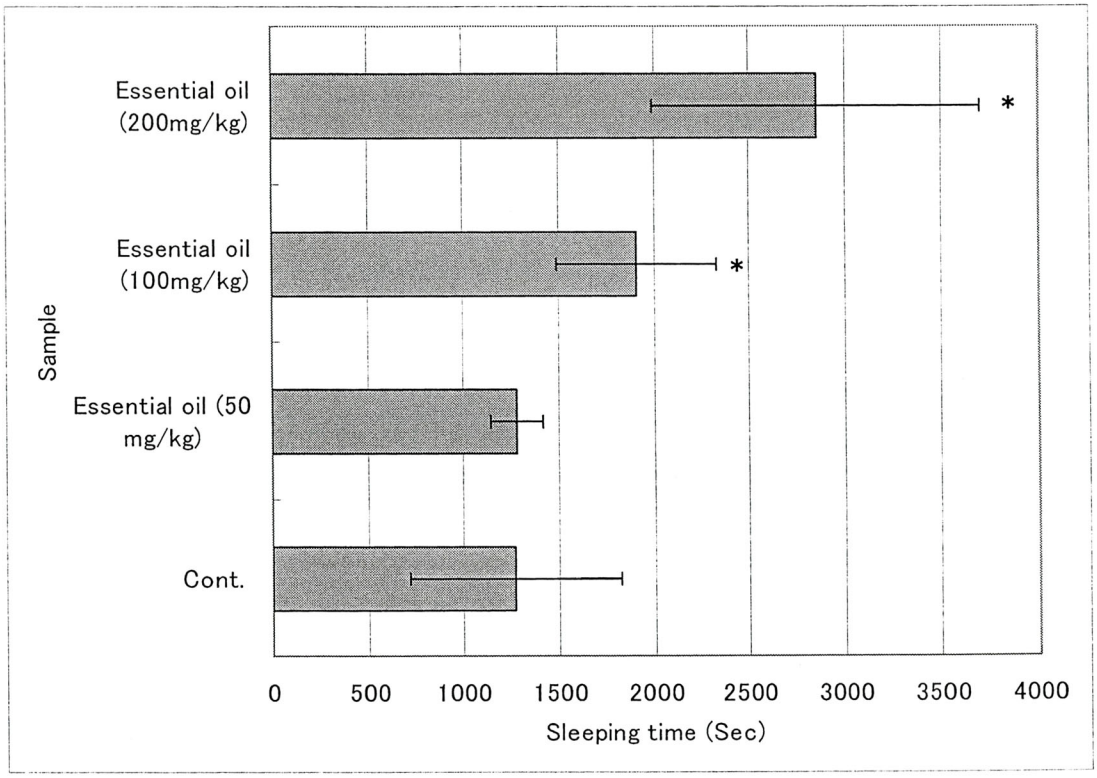


Figure 4.12. Dose-dependent effects of essential oil of *Cryptomenia japonica* on pentobarbital-induced sleeping time in mice. Pentobarbital (30 mg/kg) was injected into mice intraperitoneally 30 min after intraperitoneal injection of the sample (50 - 200 mg/kg). *P<0.05, Student's t-test for comparison between the mean values of the sample and those of the control.

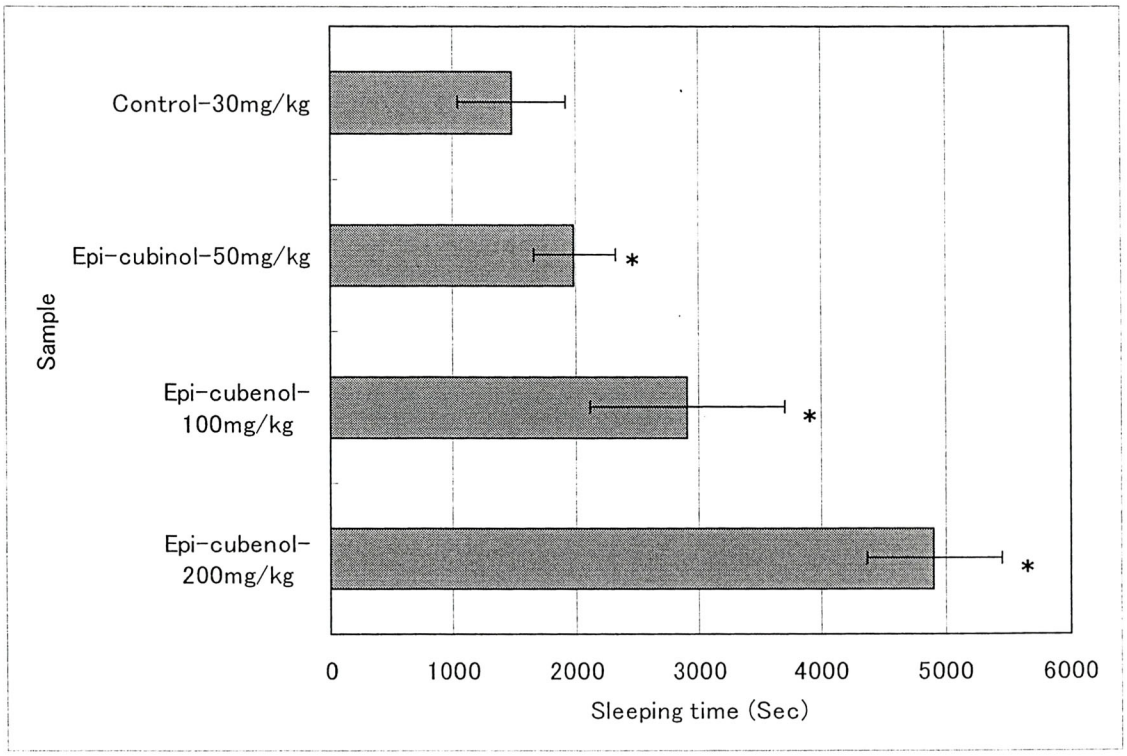


Figure 4.13. Dose-dependent effects of the fragrance compound epi-cubinol on pentobarbital-induced sleeping time in mice. Pentobarbital (30 mg/kg) was injected into mice intraperitoneally 30 min after intraperitoneal injection of the sample (50 - 200 mg/kg). *P<0.05, Student's t-test for comparison between the mean values of the sample and those of the control.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND PROSPECT

5.1. Discussion:

The present results suggest that several tested plant extracts have some antioxidant, anti-allergic and tranquillizing activity. Since a variety of constituents are known from the extracts studied, it becomes difficult to ascribe the beneficial properties selectively to any one group of constituents without further studies which are beyond the scope of this studied. Thus further extensive investigations are necessary to find out the active principles present in these plants. **Table 6** and **Figure 5.1** mention some reported bioactive compounds and bioactivity of the promising samples of the study.

Table 6: Phytochemical constituents and reported bioactivity of the promising plant samples.

Promising samples	Reported bioactive compound	Reported bioactivity	Reference
Sonneratia caseolaris Linn	Luteolin and Luteolin 7-*O*-*β*-glucoside	Antioxidant	Sadhu *et al.*, 2007
Terminalia bellirica Roxb	Gallic acid, Ellagic acid, vitamin C, catechin	Antioxidant, anti-allergic, anti-inflammatory, anti-microbial, anti-mutagenic activity	Ghoni 2003; Sabu *et al.*, 2008
Syzygium cumini Linn	Gallic acid, Ellagic acid, triterpenoids, flavonoids, tannin, alkaloids, vitamin A, C, B_1, B_2	Antioxidant, anti-diabetic, anti-allergic, anti-bacterial, anti-nociceptive activity	Rao and Roa 2001; Ghoni 2003; Banerjee *et al.*, 2005; Avila-Peno *et al.*, 2007
Terminalia arjuna Roxb	Gallic acid, Ellagic acid, Luteolin, terpenoid, saponins, phytosterol	Antioxidant, anti-allergic, anti-microbial, hypocholesterolaemic effect	Ghoni 2003; Patnaik *et al.*, 2007
Avicenia officinalis Linn	No report	No report	
Citrus paradise Mackfady.	Ascorbic acid, carotene, limonene, polyphenol like hesperidine, fibers	Antioxidant, anti-allergic activity	Bocco *et al.*, 1998; Gorinstein *et al.*, 2001

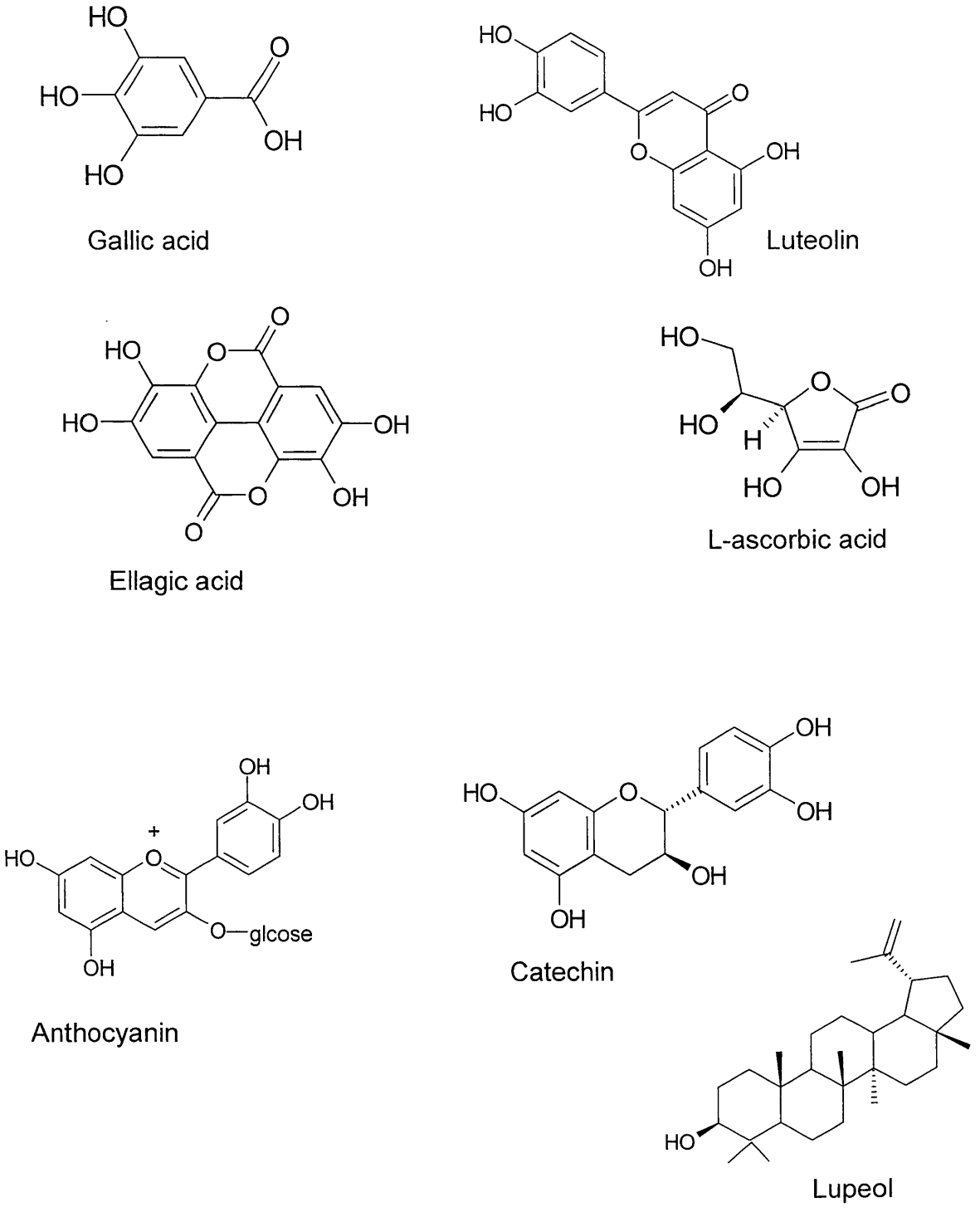


Fig.5.1. Chemical structure of some reported bioactive compounds of plant origin.

5.2. Conclusion

Bangladesh is furnished with luxuriant vegetation including the world's largest mangrove vegetation which mainly found in Sundarban, a great tidal mangrove swamp at the southwestern corner of the country. Twelve samples which are widely available and therapeutically important medicinal plants were evaluated in present study. The six samples were mangrove species and the other six were commonly used as medicinal plants. Alcohol extracts of these samples were used for the measurements of their phenolic content, DPPH radical scavenging activity, reducing power, anti-allergic activity and tranquilizing affect.

Mangrove plants examined in this study are used as folk medicine in Bangladesh had considerable amount of polyphenols and antioxidative and anti-allergic activities with *Sonnratia caseolaris* had high polyphenols and antioxidative activity whereas *Avicennia officinalis* showed strong antihistamine release effect. So the leave of *S. caseolaris* could be a good source of antioxidant phenolics and the leaves of *A. officinalis* especially its chloroform fraction would be a potential source of anti-histamine natural product.

Among the medicinal plant samples, we found that *T. arjuna* contained highest total polyphenols as well as antioxidative activities. The grading of the top polyphenol containing plant samples are: *T. arjuna* > *T. chebula* > *E. officinalis* > *T. bellirica* > *S. cumini*. These samples also showed very high anti-oxidative activity. Except *D. indica* all other extracts inhibited the release of histamine from the peritoneal exudates cells. Among them, *S. cumini* had the strongest effect. These extracts therefore have activities beneficial to physiological health.

The aqueous extracts of citrus peels had beneficial activities such as antioxidative, anti-hydrogen peroxide and anti-allergic effects and suggest a feasible application in developing anti-allergic food and supplements. Moreover, the extracts of *Citrus* peel can be used as an herbal based therapy for the treatment of allergic diseases.

Significant potentiation of the response of $GABA_A$ receptors and elongation of pentobarbital-induced sleeping time in mice caused by *S. caseolaris, T. bellirica, S. cumini,* and *T. arjuna* extracts are the prove of their tranquilizing activities. These observations suggest that these extracts may serve as sources of new supplements for improvement of mental condition. Essential oils, used in this study also showed potential regarding the development of a supplement with tranquilizing and sleep-inducing effects, which are also beneficial for mental health.

The obtained data allow mutual comparison of examined species and their assessment as possible sources of antioxidants and anti-allergic substances and give scientific support for the use of these plants in traditional medicine. All the effective results render them suitable as potential therapeutics and make them excellent candidates for more detailed investigation. Since no compositional analysis of these extracts was performed, further study is required to know the phenolic and/or nonphenolic compound(s) responsible for the anti-oxidative, anti-allergic activities and the potentiation of $GABA_A$ receptors response. **Table 7** shows the beneficial activity of the samples used in this study.

Table 7: Summarize results of the samples used in this study.

Name of the plant	Anti-oxidative activity	Anti-allergic activity	Potentiation of $GABA_A$ receptor response
Avicenia officinalis Linn	low	high	low
Derris uliginosa	low	moderate	no effect
Hibiscus tiliacious Linn	low	moderate	Very low
Manilkara zapota (L.) Royen	low	moderate	Very low
Swietenia mahagoni	low	low	Very low
Sonneratia caseolaris Linn	high	low	high
Embelica officinalis	high	high	low
Terminalia chebula (Gaertn.)	high	moderate	low
Terminalia arjuna Roxb	high	high	moderate
Terminalia bellirica Roxb	high	high	high
Syzygium cumini Linn	high	high	high
Dillenia indica Linn	high	low	low
Citrus sinensis Osbeck	high	high	ND
Citrus paradise Mackfady.	moderate	high	ND
Citrus aurantifolia Christm	high	moderate	ND
Citrus limon Burm.f	high	high	ND
Eugenia caryophyllus	ND	ND	high
Coriandrum sativum	ND	ND	moderate
Sample A (mixture of 20% *coriandrum*, 50% *Juniperus*, 20% *Abies balsamea* and 10% *Cinamomum*)	ND	ND	high
Sample B (mixture of 70% *Jasminum*, 45% *Citrus*, 45% *Cedrus* and 3% *Eugenia*)	ND	ND	high

5.3. Prospect of the study

Identification of new medicine from the medicinal herbs and plants or traditional food supplements of Bangladesh would be worthy for Japan and Bangladesh as well as for the global community. The results support the possibility of these plants, which are commonly found in Bangladesh, as a potential source and can contribute to physical and mental health. It is necessary to clarify the extract components responsible for these activities and examination of these medicinal plants for other beneficial effects is considered worthwhile. However, further *in vitro* and *in vivo* studies are needed to confirm the present observations. A multi-method approach is necessary to ensure the effectiveness of such antioxidative activity. Further studies are needed to determine whether effective extract prevents or reduces allergic symptoms in humans. After identification of novel active compounds, their derivatives must be synthesized to find more effective ones. Some *in vivo* studies are needed for better understanding their mechanism of action as antioxidant. The extracts or components active to the receptors can be examined by the mouse behavior tests to prove their effects on mood. Attention should be paid to potential cytotoxic effects when they are used for the preparation of dietary supplements.

LITERATURE CITED

Abdille, H.Md., Singh, R.P., Jayaprakasha, G.K. and Jena, B.S. (2004). Antioxidant activity of the extracts from *Dillenia indica* fruits. *Food chem.*, **90,** 891-896.

Ames, B.N. (1983). Dietary carcinogens and anticarcinogens: oxygen radicals and degenerative diseases. *Science* **221**, 1256-1264.

Ames, B.N., Shigenaga, M.K. and Hagen, T.M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Nat. Acad. Sci. USA* **90,** 7915-7922.

Aoshima, H. and Hamamoto, K. (1999). Potentiation of $GABA_A$ receptors expressed in *Xenopus* oocytes by perfume and phytoncid. *Biosci. Biotechnol. Biochem.,* **63**, 743-748.

Aoshima, H., Hossain, S.J., Hamamoto, K., Yokoyama, T., Yamada, M. and Shingai, R. (2001). Kinetic analyses of alcohol-induced potentiation of the response of $GABA_A$ receptors composed of α_1 and β_1 subunits. *J. Biochem.*, **130**, 703–709.

Aoshima, H., Okita, Y., Hossain, S.J., Fukue, K., Mito, M., Orihara, Y., Yokohama, T., Yamada, M., Kumagai, A., Nagaoka, Y., Uesato, S. and Hara,Y. (2005). Effect of 3-*O* -octanoyl-(+)-catechin on the responses of $GABA_A$ receptors and Na^+/glucose cotransporters expressed in *Xenopus* oocytes and on the oocyte membrane potential. *J. Agric. Food Chem.*, **53**, 1955-1959

Aoshima, H., Takeda, K., Okita, Y., Hossain, S.J., Koda, H. and Kiso, Y. (2006). Effects of beer and hop on ionotropic γ-aminobutyric acid receptors. *J. Agric. Food Chem.*, **54**, 2514–2519.

Aoshima, H., Tsunoue, H., Koda, H. and Kiso, Y. (2004). Aging of whiskey increases 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity. *J. Agric. Food Chem.,* **52,** 5240-5244.

Aoshima, H. and Ayabe, S. (2007). Prevention of the deterioration of polyphenol-rich beverages. *Food Chem.*, **100**, 350-355.

Ayabe, S. and Aoshima, H. (2007). Aqueous extract of citrus peel reduces production of hydrogen peroxide in catechin-enriched green tea. *Food Chem.*, **104**, 1594-1598.

Balasooriya, S. J., Sotheeswaran, S., and Balasubramanium, S. (1982). Economically useful plants of Sri Lanka, Part IV. *J. Nat. Sci. Counc.*, **10**, 213-219.

Baur, R., Simmen, U., Senn, M., Sequin, U. and Sigel, E., (2005). Novel plant substances acting as beta subunit isoform-selective positive allosteric modulators of $GABA_A$ receptors. *Mol. Pharmacol.* **68**, 787-792.

Banerjee, D., Chakrabarti, S., Hazra, K.A., Banerjee, S., Ray, J. and Mukherjee B. (2008). Antioxidant activity and total phenolics of some mangroves in Sundarbans. African J. of Biotech., **7(6),** 805-810.

Blois M.S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature,* **181,** 1199-1200.

Blackham, A. and Woods, F.A. (1986). Immune complex mediated inflammation in the mouse peritoneal cavity. A method for investigating inhibitors of arachidonate metabolism. *J. Pharmacol. Methods*, **15**, 77-85.

Bocco, A., Cuveilier, M.E., Richard, H. and Berset, C. (1998). Antioxidant activity and phenolic composition of citrus peel and seed extract. *J. Agric. Food Chem.*, **46**, 2123-2129.

Butlet, M.S. (2004). The role of natural product chemistry in drug discovery. *J. Nat. prod.* **67**, 2141-2153.

Cai, Y., Luo, Q., Sun, M. and Corke, H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci.* **74**, 2157–2184.

Chanwitheesuk, A., Teerawutgulrag, A. and Rakariyatham, N. (2005). Screening of antioxidant activity and antioxidant compounds of some edible plants of Thailand. *Food Chem,*. **92**, 491–497.

Chebib, M. and Johnston, G.A.R. (2000). GABA-activated ligand gated ion channels: medicinal chemistry and molecular biology. *J. Med. Chem.*, **43**, 1427-1447.

Corvazier, E. and Maclouf, J. (1985). Interference of some flavonoids and non-steroidal anti-inflammatory drugs with oxidative metabolism of arachidonic acid by human platelet and neutrophils. *Biochim. Biophys. Acta.*, **835**, 315-321.

Dekkers, J.C., Doornen L.J.P. and Han C.G. (1996). The role of antioxidant vitamins and enzymes in the prevention of exercise-induced muscle damage. *Sports Med.*, **21,** 213-238.

Di Matteo, V. and Esposito, E. (2003). Biochemical and therapeutic effects of antioxidants in the treatment of Alzheimer's disease, Parkinson's disease, and qmyotrophic lateral sclerosis. *Curr. Drug Targets-CNS & Neurol. Disorders*, **2**, 95-107.

Duh, P.D., Tu, Y.Y. and Yen, G.C. (1999). Antioxidant activity of water extract of Harng Tyur (*Chrysanthemum morifolium* Ramat.). *Lebensm. Wiss Technol.* **32,** 269-277.

Fujimura, Y., Umeda, D., Yamada, K. and Tachibana, H. (2008). The impact of the 67kDa laminin receptor on both cell-surface binding and anti-allergic action of tea catechins. *Arch. Biochem. Biophys.*, **476**, 133-138.

Fujimura, Y., Umeda, D., Yano, S., Maeda-Yamamoto, M., Yamada, K. and Tachibana, H. (2007). The 67kDa laminin receptor as a primary determinant of anti-allergic effects of O-methylated EGCG. *Biochem. Biophys. Res. Commun.*, **364,** 79-85.

Garcia, D.A., Bujons, J., Vale, C. and Sunol, C. (2006). Allosteric positive interaction of thymol with the $GABA_A$ receptor in primary cultures of mouse cortical neurons. *Neuropharmaco.* **50**, 25-35.

Goni, A. (2003). "Medicinal Plants of Bangladesh with Chemical Constituents and Uses." 2nd ed. Asiatic Society of Bangladesh, Dhaka.

Gorinstein, S., Martin-Belloso, O., Park, Y.S., Haruenkit, R., Lojek, A., Ciz, M., Capsi, A., Libman, I. and Trakhtenberg, S. (2001). Comparioson of some biochemical characteristics of different citrus fruits. *Food Chem.*, **74**, 309-315.

Granger, R.E., Campbell, E.L. and Johnston, G.A. (2005). (+)- And (-)-borneol: efficacious positive modulators of GABA action at human recombinant alpha 1 beta 2 gamma 2L GABA(A) receptors. *Biochem. Pharmacol.* **69**, 1101-1111.

Gunther, U., Benson, J.A., Benke, D., Fritschy, J.M., Reyes, G., Knoflach, F., Cretani, F., Aguzzi, A., Arigoni, M., Lang, Y., Mohler, H. and Luscher, B. (1995). Benzodiazepine-insensitive mice generated by targeted disruption of the γ_2 subunit gene of γ-aminobutyric acid A receptors. *Proc. Natl. Acad. Sci. USA* **92**, 7749-7753.

Gulcin, I., Elmastat, M. and Y. Aboul-Enein H. (2007). Determination of antioxidant and radical scavenging activity of Basil (*Ocimum basilicum L.* Family Lamiaceae) assayed by different methodologies. *Phytother. Res.* **21,** 354-361.

Harrison, N.L., Krasowski, M.D. and Harris, R.A. (2000). "Alcohol and general anesthetic actions on the $GABA_A$ receptor". In "GABA in the Nervous System: The View at Fifty Years," ed. by D. L. Martin and R.W. Olsen. Lippincott Wikkuams & Wilkins, Philadelphia, pp 167-189.

Hannum, S.M. (2004). Potential impact of strawberries on human health: a review of science. *Curr. Rev. Sci. Nutri.*, **44,** 1-17.

Hayakawa, K., Kimura, M. and Kamata, K. (2002). Mechanism underlying γ-aminobutyric acid-induced antihypertensive effect in spontaneously hypertensive rats. *Eur. J. Pharmacol.*, **438**, 107-113.

Hossain, S.J., Aoshima, H., Koda, H. and Kiso, Y. (2002a). Potentiation of the ionotropic GABA receptor response by whiskey fragrance. *J. Agric. Food Chem.*, **50**, 6828–6834.

Hossain, S.J., Aoshima, H., Koda, H. and Kiso, Y. (2003). Effects of coffee components in the response of $GABA_A$ receptors expressed in *Xenopus* oocytes. *J. Agric. Food Chem.*, **51**, 7568-7575.

Hossain, S.J., Aoshima, H., Koda, H. and Kiso, Y. (2004). Fragrances in oolong tea that enhance the response of $GABA_A$ receptors. *Biosci. Biotechnol. Biochem.*, **68**, 1842-1848.

Hossain, S.J., Aoshima, H., Koda, H. and Kiso, Y. (2007). Review of functional studies of beverage components acting on the recombinant $GABA_A$ neuroreceptor, and Na^+/glucose cotransporter-response using the *Xenopus* oocyte expression system and electrophysiological measurements. *Food Biotechnol.*, **21**, 237-270.

Hossain, S.J., Hamamoto, K., Aoshima, H. and Hara, Y. (2002b). Effects of tea components on the response of $GABA_A$ receptors expressed in *Xenopus* oocytes. *J. Agric. Food Chem.*, **50**, 3954–3960.

Hossain, S.J., Kato, H., Aoshima, H., Yokoyama, T., Yamada, M. and Hara,Y. (2002c). Polyphenol-induced inhibition of the response of Na^+/glucose cotransporter expressed in *Xenopus* oocytes. *J. Agric. Food Chem.*, **50**, 5215-5219.

Hossain, S.J., Tsujiyama, I., Takasugi, M., Islam, M.A., Biswas, R.S. and Aoshima, H. (2008). Total phenolic contents, antioxidative, anti-amylase, anti-glucosidase, and antihistamine release activities of Bangladeshi fruits. *Food Sci. Technol. Res.*, **14**, 261-268.

Ho, A.C. and Lon, C.C. (2008). Investigation of heat treating conditions for enhancing the anti-inflammatory activity of citrus fruit (*Citrus reticulata*) peels. *J. Agri. Food Chem.*, **56**, 7976-7982.

Hui, K.M., Huen, M.S., Wang, H.Y., Zheng, H., Sigel, E., Baur, R., Ren, H., Li, Z.W., Wong, J.T. and Xue, H. (2002). Anxiolytic effect of wogonin, a benzodiazepine receptor ligand isolated from Scutellaria baicalensis Georgi. *Biochem. Pharmacol.*, **64**, 1415-1424.

Itoh, T., Ohguchi, K., Iinuma, M., Nozawa, Y. and Alao, Y. (2008). Inhibitory effects of polymethoxy flavones isolated from *Citrus reticulate* on degranulation in rat basophilic leukemia RBL-2H3: enhanced inhibition by their combination. *Bioorg. Med. Chem.*, **16**, 7592-7598.

James, R.H. (2003). Natural product: the secondary metabolites. Royal Society of Chemistry.

Kaliner, M., Shelhamer, J.H. and Ottesen, E.A. (1982). Effects of infused histamine: correlation of plasma histamine levels and symptoms. *J. Allergy and Clin. Immunol.*, **69**, 283-289. .

Kanda, T., Akiyama, H., Yanagida, A., Tanabe, M., Goda, Y., Toyoda, M., Teshima, R. and Saito,Y. (1998). Inhibitory effects of apple polyphenol on induced histamine release from RBL-2H3 cells and rat mast cells. *Biosci. Biotechnol. Biochem.*, **62**, 1248-1289.

Kaneko, Y., Motohashi, Y., Nakamura, H., Endo, T. and Eboshiba, A. (2005). Increasing prevalence of Japanese ceder pollinosis: a meta-regression analysis. *Int. Arch. Allergy Immunol.* **136**, 365-371.

Kawai, M., Hirano, T., Higa, S., Arimitsu, J., Maruta, M., Kuwahara, Y., Ohkawara, T., Hagihara, K., Yamadori, T., Shima, Y., Ogata, A., Kawase, I. and Tanaka, T. (2007). Flavonoids and related compounds as anti-allergic substances. *Allergol. Int.* **56**, 113-123.

Kirtikar, K.R. and Basu, B.D. (1987). "Indian medicinal plants, 2nd ed.". International Book Distributors, India.

Koda, H., Hossain, S.J., Kiso, Y. and Aoshima, H. (2003). Aging of Whiskey increases the potentiation of $GABA_A$ receptor response. *J. Agric. Food Chem.*, **51**, 5238-5244.

Kusano, K., Miledi, R. and Stinnarkre, J. (1982). Cholinergic and catecholaminergic receptors in the *Xenopus* oocytes membrane. *J. Physiol.*, **328**, 143–170.

Kim, S.H, Jun, C.D., Suk, K., Choi, B.J., Lim, H., Park, S., Lee, S.H., Shin, H.Y., Kim D.K., Shin, T.Y. (2006) Gallic acid inhibits histamine release and pro-inflammatory cytokine production in mast cells. *Toxicol. Sci.* **91,** 123-131.

Kris-Etherton, P.M., Hecker, K.D., Bonanome, A., Coval, S.M., Binkoski, A.E., Hilpert, K.F., Griel, A.E. and Etherton, T.D. (2002). Bioactive compounds in foods: their role in the prevention of cardiovascular diseases and cancer. *Am. J. Med.*, **113**, 71S-88S.

Li, S., Lo, C.Y., and Ho, C.T. (2006). Hydoroxylated polymethoxyflavones and methylated flavonoids in sweet orange (*Cutrus sinesis*) peel. *J. Agri. Food Chem.*, **54**, 4176-4185.

Marks, D.R. and Marks, L.M. (1993). Food allergy. *Postgrad. Med.*, **93**, 191-201.

Matsuo, N., Yamada, K., Mori, M., Shoji, K., Ueyama, T., Yunoki, S., Yamashita, K., Ozeki, M. and Sugano, M. (2000). Inhibition by dietary tea polyphenols of chemical mediator release from rat peritoneal exudates cells. *Biosci. Biotechnol. Biochem.*, **64**, 1437-1443.

Mihic, S.J., Ye, Q., Wick, M.J., Koltchine, V.V., Krasowski, M.D., Finn, S.E., Mascia, M.P., Valenzuela, C.F., Hanson, K.K., Greenblatt, E.P., Harris, R.A., and N.L. (1997). Sites of alcohol and volatile anesthetic action on $GABA_A$ and glycine receptors. *Nature*, **389**, 385-389.

Mitou, M., Shigemori, Y., Aoshima, H., and Yokoyama, S. (2008). Effect of dried bonito (katsuobushi) and some of its components on $GABA_A$ receptors. *Food Chem.*, **108**, 840-846.

Misra PM and Sree A. (2007). Antibacterial activity and GCMS analysis of the extract of leaves of *Finlaysonia obovata* (A mangrove plant). *Asi. J. of plant sci.* **6**(1), 168-172.

Mubassara, S., Hossain, S. J., Ahmed, F., Yamamoto, M., Tan, N., and Aoshima, H. (2009). Potentiation of the response of $GABA_A$ receptors by Bangladeshi medicinal plants. *Food Sci. Technol. Res.*, **15**, 315-324.

Murakami, A., Nakamura, Y., Torikai, K., Tanaka, T., Koshiba, T., Koshimizu, K., Kuwahara, S., Takahashi, Y., Ogawa, K., Yano, M., Tokuda, H., Nishino, H., Mimaki, Y., Sashida, Y., Kitanaka, S., and Ohigashi, H. (2000). Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. *Cancer Res.*, **60**, 5059-5066.

Nakatani N. (2000). Phenolic antioxidants from herbs and spices. *BioFactors* 13, 141-146.

Nicholls, D.G., (1994). "Proteins, Transmitters and Synapses" Blackwell Scientific Publications, Oxford, pp 155-199.

Oyaizu M. (1986). Studies on products of browning reaction: antioxidative activity of products of browning reaction prepared from glucosamine. *Jpn. J. Nutr.* **44**, 307-315.

Park, H.H., Lee, S., Son, H.Y., Park, S.B., Kim, M.S., Choi, E.J., Singh, T.S.K., Ha, J.H., Lee, M.G., Kim, J.E., Hyun, M.C., Kwon, T.K., Kim, Y.H. and Kim, S.H. (2008). Flavonoids inhibit histamine release and expression of proinflammatory cytokines in mast cells. *Arch. Pharma. Res.*, **31**, 1303-1311.

Perez, R.M., Perez, J.A., Garcia, L.M. and Sossa, H. (1998) Neuropharmacological activity of *Solanum nigrum* fruis, *J. Ethnopharmacol.*, **62**, 43-48

Pokorny, J. (1991). Natural antioxidant for food use. Trends in food sci. and technol., **2**, 223-227.

Rice-evans, C.A., Miller, N.J., Bolwell, P.G., Bramley, P.M. and Pridham, J.B. (1995). The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Rad. Res.*, **22(4)**, 375-383.

Robinson, C. and Holgate, S.T. (1985). New perspectives on the putative roles of eicosanoids in airway hyper-responsiveness. *J. Allergy Clin. Immunol.*, **76,** 140-144.

Sadhu, S.K., Ahmed, F., Ohtsuki T. and Ishibashi M. (2006). Flavonoids from *Sonneratia caseolaris*. *J. Natural Med.*, **6**, 264-265.

Scartezzini, P., Antognoni, F., Raggi, M.A., Poli, F. and Sabbioni, C. (2006). Vitamin C content and antioxidant activity of the fruit and of the Ayurvedic preparation of *Emblica officinalis* Gaertn. *J. Ethnopharmacol.*, **104**, 113-118.

Shon, M.Y., Cho, S.D., Kahng G.G., Nam S.H. and Sung, N.J. (2004). Antimutagenic, antioxidant and free radical scavenging activity of ethyl acetate extracts from white, yellow and red onions. *Food Chem. Toxicol.*, **42,** 659-666.

Shore, P.A., Burkhalter A. and Cohn V.H.A. (1959). A method for the fluorometric assay of histamine in tissues. *J. Pharmacol. Exp. Ther.*, **127,** 182-186.

Sieghart, W. (1995). Structure and pharmacology of gamma-aminobutyric acid A receptor subtypes. *Pharmacol. Rev.*, **47**, 181-234.

Tachibana, H., Koga, K., Fujimura, Y. and Yamada, K. (2004). A receptor for green tea polyphenol EGCG. *Nat. Structure Mol. Biology*, **11**, 380-381.

Takasugi, M., Kitagawa, T., Kato, M., Maeda, N., Nagata, N., Niwa, J. and Shimada, N. (2008). Correlation among suppression of leukotriene B_4 release, radical scavenging activity and polyphenol contents of commercial tea extract. *Nippon Shokuhin Kagaku Kogaku Kaish*, **55**, 87-94.

Takasugi, M., Shimada, K. and Yamada, K. (2002). Studies on inhibitory effect of soy isoflavones on release of allergy-related mediators from rat peritoneal exudates cells. *Soy Protein Res.*, **5**, 99-102.

Trauner, G, Khom, S., Baburin, I., Benedek, B., Hering, S. and Kopp, B. (2008). Modulation of $GABA_A$ receptors by Valerian extracts is related to the content of Valerenic acid, *Planta Med.*, **74**, 19-24

Umezu, T. (1999). Anti-conflict effects of plant-derived essential oils. *Pharmacol. Biochem. Behave.*, **64**, 35-40.

Vinson, J.A., Proch, J., and Bose, P. (2001). Determination of quantity and quality of polyphenol antioxidants in food and beverages. *Methods Enzymol.*, **335**, 103-114.

Whiting, P.J., Wafford, K.A. and McKernan, R.M. (2000). "Pharmacologic subtypes of $GABA_A$ receptors based on subunit composition". In "GABA in the Nervous System: The View at Fifty Years," ed. by D. L. Martin and R.W. Olsen. Lippincott Wikkuams & Wilkins, Philadelphia, pp 113-126.

Younginger JW. (1992). Anaphylaxis. *Ann Allergy*, **69,** 87-96.

題目：植物資源からの有用生理活性の探索

社会の高齢化やストレスの増加に伴い、心身の健康改善に役立つサプリメントの開発が望まれている。そのためサプリメントや薬の原料となる新しい植物原料を見つけることが重要である。東南アジアのバングラデシュにある５千の顕花植物の千を越えるものは、有用な化学成分を持つと考えられる。これらのいろいろの植物は伝統的に民間薬として使われてきた。そこで多くのバングラデシュ産の植物や果物をアルコール抽出し、心身の健康に有用な効果を明らかにするために細胞系および非細胞系で生物活性を研究した。

すでに１２種類のバングラデシュ産果物のいくつかに有用な活性を見つけたため、この研究ではまずバングラデシュ産の６種類のマングルーブのアルコール抽出物の総フェノール量、抗酸化活性、ヒスタミン放出抑制活性を測定した。総ポリフェノール量はフォーリンーチオカルト法により測定し、抗酸化活性はDPPHラジカル補足活性と還元力により評価した。これらの値の間には、高い相関が見られた。*S. seolaris*は、最も強いDPPHラジカル捕捉活性（IC_{50} = 18 μg/mL）を示した。また他の５つの試料もポリフェノールを含み強い抗酸化活性を示した。

６種類のマングルーブ抽出物の抗アレルギー活性を、ラットの腹腔内滲出細胞を用いて検討した。Ca^{2+}イオノフォアのA23187によって滲出細胞から放出されるヒスタミンの量を蛍光分光法により測定し、抽出試料による放出抑制を測定した。測定した全ての試料は滲出細胞からのA23187によるヒスタミンの放出を抑制し、抗アレルギー活性を示した。これらの試料のなかで、*A. officinalis*が最も強い効果（IC_{50} = 0.5 μM GAE）を示した。以前の研究で柑橘類の表皮が、茶カテキン類からの過酸化水素生成を抑制した。そこで複数の柑橘類表皮の抽

出液のヒスタミンの放出抑制とマウスの腹腔内の炎症による色素の滲出抑制を測定し、抗アレルギー活性を評価した。全ての測定した柑橘類の表皮抽出液はヒスタミンの滲出細胞からの放出を抑制し、この抑制は抽出液を１００℃で１０分間過熱することで強まった。グレイプフルーツ表皮の抽出液を経口投与したところ、色素の滲出によって測定した腹腔内の酢酸による炎症は抑制された。

活性成分の性質を明らかにするために、高い抗酸化活性とヒスタミン放出活性を示した*S. caseolaris*と*A. officinalis*の抽出物を様々な溶媒で抽出した。*S. caseolaris*は酢酸エチルアとエタノールの分画に高い抗酸化活性が見られた。はクロロホルムの分画が強いヒスタミン放出抑制を示し、ヘキサンや酢酸エチル分画にも抑制活性が見られた。

イオンチャネル型ガンマアミノ酪酸受容体（$GABA_A$受容体）は脳内の主要な抑制性の神経伝達物質の受容体である。$GABA_A$受容体応答を昂進すると、ベンゾジアゼピンなどの薬物で見られるように、鎮静効果が生じることが知られている。また$GABA_A$受容体応答を昂進する化合物を投与すると、ペントバルビタールなどにより誘導される睡眠時間を延長することが予想される。なおペントバルビタールは$GABA_A$受容体応答を昂進して睡眠薬として働くことが知られている。シナモン、コリアンダーやクローブなどの精油やそのブレンド精油は、アフリカツメガエル卵母細胞に発現させた$GABA_A$受容体応答を昂進させた。これらの精油を腹腔投与あるいは吸気を通してマウスに投与して、ペントバルビタールで誘導される睡眠時間への影響を測定した。どちらの投与方法でも精油はマウスの睡眠時間を延長させた。これらの精油は吸うと$GABA_A$受容体に作用して気分をリラックスさせる可能性がある。鎮静効果をもたらす抽出物を探すために、アフリカツメガエル卵母細胞に$GABA_A$受容体を発現させ、１２種類のバングラデシュ産の果物や植物の抽出物の効果を測定した。*S. caseolaris*、 *T. bellirica*、 *S. cumini*

や*T. arjuna* の抽出物は、これら自身は応答を示さなかったが、有意に $GABA_A$ 受容体応答を昂進させた。これらの抽出物をマウスに腹腔あるいは経口投与したところ、ペントバルビタールによる睡眠時間を延長させた。$GABA_A$ 受容体応答の昂進とペントバルビタールによる睡眠時間の延長の間には、高い相関（$r = 0.92$）が見られた。また両者は抽出物のきれいな濃度依存性を示した。

結論として、バングラデシュ産のマングローブのアルコール抽出物は多量のポリフェノールを含み、高い抗酸化活性とヒスタミン放出抑制を示したことから、健康食品を開発するのに役立つ可能性がある。柑橘類の抽出液もヒスタミン放出を抑制し、抗アレルギー活性が期待される。バングラデシュ産の果物やマングローブの複数の抽出物は、いくつかの精油と同様に細胞系および非細胞系において $GABA_A$ 受容体に作用した。これらの抽出物は精神に有用な鎮静作用や睡眠誘導作用を持ったサプリメントの開発に役立つと思われる。

Major publications

I have published the following papers during my tenure:

Published papers:

1. **Sanzida Mubassara,** Ushijima Asumi, Tan Nobusuke, and Aoshima Hitoshi. Effect of essential oils on the response of $GABA_A$ receptors and sleeping time in the mice induced by a sleeping drug. ***Aroma Research*,No. 35 9(3), 348-353 (2008), (Japan).**

2. **Sanzida Mubassara,** Sheikh Julfikar Hossain, Firoj Ahmed, Makie Yamamoto,Nobusuke Tan and Hitoshi Aoshima. Potentiation of the response of $GABA_A$ receptors by Bangladeshi Medicinal plants. ***Food Science and Technology Research,* 15(3), 315-324 (2009).**

ADDITIONAL PUBLICATION

1. Akond, M.A., Saidul Alam, S.M.R. Hasan, **Sanzida Mubassara**, Sarder Nasir Uddin, and Momena Shirin. 2009. Bacterial contaminants in Carbonated Soft Drinks sold in Bangladesh markets. ***International J. Food Microbiology*** **130**(2): 156-158. (Official journal of the International Union of Microbiological Societies (IUMS) and the International Committee on Food Microbiology and Hygiene (ICFMH). ELSEVIER, UK.

2. Akond, M.A., **S. Mubassara,** and M.M. Rahman. 2007. Effects of inorganic salts on growth and respiratory activity of *Azospirillum* spp. isolated from wheat fields of Bangladesh. ***J. Asiatic Society Bangladesh, Science*** **33**(2): 215-222. Asiatic Society, Bangladesh.

3. Akond, M.A., **S. Mubassara** and M.M. Rahman. 2007. Distribution and abundance of *Azotobacter* in wheat fields of Bangladesh. ***Bangladesh J. Microbiology*** **24**(2): 151-153. Bangaldesh Society of Microbiologists.

4. Rahman, M.M., **Mubassara, S.,** Hoque, S. and Khan, Z.U.M. 2007. Effect of *Azospirillum* inoculation on growth and yield of lentil. *Bangladesh J. Microbiology.* **24** (1): 30-33.

5. Rahman, M.M., **Mubassara, S.,** Hoque, S. and Khan, Z.U.M. 2006. Aerobic heterotrophic bacteria and *Azospirillum* in certain saline soils of Bangladesh. *Bangladesh J. Life Sci.* **18**(1):105-110.

6. Rahman, M.M., **Mubassara, S.**, Hoque, S. and Khan, Z.U.M. 2006. Effect of some environmental factors on the growth of *Azospirillum* species isolated from saline soils of Satkhira District, Bangladesh. *Bangladesh J. Microbiology.* **23**(2): 145-148.