Appendix A

LIST OF CHEMICALS USED

CHEMICALS USED

A

ABTS

Acetic anhydride

Acetone

Aluminium chloride

Ammonia

Antimony chloride

Arnow's reagent

B

Benzene

Bismuth subnitrate

Butanol

C

Carbon tetrachloride

Carrageenan

Chloroform

Citrate buffer

Copper sulphate

D

Diethyl ether

DPPH

Dragendorff reagent

E

Ethanol

Ethyl acetate

F

Ferous chloride

Ferric chloride

Ferrozine

Folin-ciocalteau reagent

Folin-denis reagent

Formic acid

G

Glacial acetic acid

Glibenclamide

Glucose

Glycerine

Greiss reagent

H

HCl

Heptane

Hexane

Hippuryl-histidyl-leucine

I

Iodine

Isopropanol

K

KOH

L

Lead acetate

Liebermann burchard’s reagent

M

Methanol

MTT

NBT

N

Nicotinamide

Ninhydrin

Nitric acid

NP/PEG

P

Petroleum benzene

Petroleum ether

Phloroglucinol

Phospho molybdic acid

Phosphoric acid

PMS

PNPG

Potassium ferricyanide

Potassium hydroxide

Potassium iodide

Potassium persulphate

S

Silica

Sodium carbonate

Sodium hydroxide

Sodium molybdate

Sodium nitrite

Sodium nitroprusside

Sodium potassium tartarate

Sodium tungstate

Streptomycin

Streptozotocin

Sudan III

Sulphuric acid

Superoxide

T

Tannic acid

Toluene

Tri-chloro acetic acid (TCA)

V

Vanillin

Appendix B

ABBREVIATIONS USED

ABBREVIATION

A

AAAc	Acetone extract of formulation AA
AAAq	Aqueous extract of formulation AA
AABu	Butanol extract of formulation AA
AABz	Benzene extract for formulation AA
AACl	Cloroform extract for formulation AA
AAEa	Ethyl acetate extract for formulation AA
AAEt	Ethanol extract of formulation AA
AAHp	Heptane extract for formulation AA
AAHx	Hexane extract of formulation AA
AAMt	Methanol extract of formulation AA
AB	Autoclave boiling
ABTS	2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ACE	Angiotensin converting enzyme
AHA	Anti-hypertensive activity
ALP	Alkaline phosphatase
AR	Formulation for arthritis
ARAc	Acetone extract of formulation AR
ARAq	Aqueous extract of formulation AR
ARBu	Butanol extract of formulation AR
ARBz	Benzene extract for formulation AR
ARCl	Cloroform extract for formulation AR
AREa	Ethyl acetate extract for formulation AR
AREt	Ethanol extract of formulation AR
ARHp	Heptane extract for formulation AR
ARHx	Hexane extract of formulation AR
ARMt	Methanol extract of formulation AR
AS	Formulation for asthma
ASAc	Acetone extract of formulation AS
ASAq	Aqueous extract of formulation AS
ASBu	Butanol extract of formulation AS
ASBz	Benzene extract for formulation AS
ASCl	Cloroform extract for formulation AS
ASEa	Ethyl acetate extract for formulation AS
ASEt	Ethanolic extract of formulation AS
ASHp	Heptane extract for formulation AS
ASHx	Hexane extract of formulation AS
ASMt	Methanolic extract of formulation AS

B

BCG	Bromocresol green
BOFF	Bark of Frainus floribunda
BP	Formulation for High blood pressure
BPAc	Acetone extract of formulation BP
BPAq	Aqueous extract of formulation BP
BPBu	Butanol extract of formulation BP
BPBz	Benzene extract for formulation BP
BPCl	Cloroform extract for formulation BP
BPEa	Ethyl acetate extract for formulation BP
BPEt	Ethanol extract of formulation BP
BPHp	Heptane extract for formulation BP
BPHx	Hexane extract of formulation BP
BPMt	Methanol extract of formulation BP

C

CB	Formulation for sinusitis
CBAc	Acetone extract of formulation CB
CBAq	Aqueous extract of formulation CB
CBBu	Butanol extract of formulation CB
CBBz	Benzene extract for formulation CB
CBCl	Cloroform extract for formulation CB
CBEa	Ethyl acetate extract for formulation CB
CBEt	Ethanol extract of formulation CB
CBHp	Heptane extract for formulation CB
CBHx	Hexane extract of formulation CB
CBMt	Methanol extract of formulation CB
CE	Catechol equivalents
CONT	Control group
COX	Cycloxygenase
CP	Cold percolation

D

DMSO	Dimethyl Sufoixide
DPPH	1,1-diphenyl-2- picrylhydrazyl

E

EW	Extractive weight

F

FF	Formulation of diabetes
FFAc	Acetone extract of formulation FF
FFAq	Aqueous extract of formulation FF
FFBu	Butanol extract of formulation FF

FFBz	Benzene extract for formulation FF
FFCl	Cloroform extract for formulation FF
FFEa	Ethyl acetate extract for formulation FF
FFEt	Ethanol extract of formulation FF
FFHp	Heptane extract for formulation FF
FFHx	Hexane extract of formulation FF
FFMt	Methanol extract of formulation FF
FP	Formulation for food poisoning
FPAc	Acetone extract of formulation FP
FPAq	Aqueous extract of formulation FP
FPBu	Butanol extract of formulation FP
FPBz	Benzene extract for formulation FP
FPCl	Cloroform extract for formulation FP
FPEa	Ethyl acetate extract for formulation FP
FPEt	Ethanol extract of formulation FP
FPHp	Heptane extract for formulation FP
FPHx	Hexane extract of formulation FP
FPMt	Methanol extract of formulation FP
FRAP	Ferric reducing antioxidant power
FWT	Fresh weight tissue

G

GAE	Gallic acid equivalent
GC-MS	Gas Chromatography- Mass Spectroscopy
GS	formulation for gastritis
GSAc	Acetone extract of formulation GS
GSAq	Aqueous extract of formulation GS
GSBu	Butanol extract of formulation GS
GSBz	Benzene extract for formulation GS
GSCl	Cloroform extract for formulation GS
GSEa	Ethyl acetate extract for formulation GS
GSEt	Ethanol extract of formulation GS
GSHp	Heptane extract for formulation GS
GSHx	Hexane extract of formulation GS
GSK-3	Glycogen synthase kinase-3
GSMt	Methanol extract of formulation GS

H

HDL	High-density lipoprotein
HP	Formulation for heart palpitation
HPAc	Acetone extract of formulation HP
HPAq	Aqueous etxract of formulation HP
HPBu	Butanol etxract of formulation HP

HPBz	Benzene extract for formulation HP
HPCl	Cloroform extract for formulation HP
HPEa	Ethyl acetate extract for formulation HP
HPEt	Ethanol etxract of formulation HP
HPHp	Heptane extract for formulation HP
HPHx	Hexane extract of formulation HP
HPMt	Methanol extract of formulation HP
HPTLC	High Performance Thin Layer Chromatography
HS	Formulation for indigestion and stomachahe
HSBu	Butanol extract of formulation HS
HSBz	Benzene extract for formulation HS
HSCl	Cloroform extract for formulation HS
HSEa	Ethyl acetate extract for formulation HS
HSAc	Acetone extract of formulation HS
HSAq	Aqueous extract of formulation HS
HSEt	Ethanol extract of formulation HS
HSHp	Heptane extract for formulation HS
HSHx	Hexane extract of formulation HS
HSMt	Methanol extract of formulation HS

K

KOH	Potassium hydroxide

L

LDL	Low-density lipoprotein

M

MC	Metal chelation
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N

NADH	Nicotinamide adenine dinucleotide hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NB	Normal boiling
NIDDM	Non-insulin dependent diabetes mellitus
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
NO_2	Nitrogen dioxide
NOR	
CONT	Normal control group

O

OD	Optical density
OGTT	Oral Glucose Tolerance Test

P

PBS	Phosphate Buffer
PCA	Principal Component Analysis
pH	Power of hydrogen
PPHG	Postprandial hyperglycemia

Q

QE	Quercetin equivalent

R

RF	Retention factor
RNS	Radical Nitrogen Species
ROS	Reactive Oxygen Species

S

S	Soxhletion
SE	Standard error
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SO	Super oxide

T

TAC	Total alkaloid content
TAE	Tannic acid equivalents
TCL	Total Cholesterol
TFC	Total flavonoid content
TGL	Triglycerides
TLC	Thin Layer Chromatography
TOPC	Total orthodihydric phenol content
TPC	Total phenol content
TS	Formulation for tonsilitis
TSAc	Acetone extract of formulation TS
TSAq	Aqueous extract of formulation TS
TSBu	Butanol extract of formulation TS
TSBz	Benzene extract for formulation TS
TSC	Tannin Steroid content
TSCl	Cloroform extract for formulation TS
TSEa	Ethyl acetate extract for formulation TS
TSEt	Ethanol extract of formulation TS
TSHp	Heptane extract for formulation TS
TSHx	Hexane extract of formulation TS
TSMt	Methanol extract of formulation TS
TTC	Total Tannin content

Appendix C

LIST OF PUBLICATIONS FROM THE THESIS

LIST OF PUBLICATIONS

A Subba, P Mandal. 2015. Antioxidant potential of *Fraxinus floribunda* Bark Extracted through Various Aqueous Processing. *Free Radicals and Antioxidants*. 5: 6-12.

A Subba, P Mandal. 2015. Pharmacognostic studies and *in vitro* antioxidant potential of traditional polyherbal formulation of West Sikkim with *Asparagus* Spp. *Pharmacognosy Journal*. 7: 348-55.

A Subba, B Dutta, RK Sahu, P Mandal. 2017. Antioxidant, anti-inflammatory, and hepatoprotective activity of *Fraxinus floribunda* bark and the influence of extraction process on their bioactivity. 11: 983-90.

A Subba, P Mandal. 2019. Standardization of a traditional polyherbal formulation with pharmacognostic study; its phytochemical content, antioxidant, and antidiabetic activity. *Asian Journal of Pharmaceutical and Clinical Research*. 12: 182-90.

A Subba, RK Sahu, Bhardwaj S, P Mandal. 2019. Alpha glucosidase inhibiting activity and *in vivo* antidiabetic activity of *Fraxinus floribunda* bark in streptozotocin-induced diabetic rats. *Pharmacognosy Research*. 11: 273-78.

SEMINARS ATTENDED

NATIONAL

National conference of Plant Physiology, December 13-16, 2013.

National symposium on Recent Trends in Plant and Microbial Research, March 22-23, 2013.

National seminar on Plant and Microbe: Diversity and Utilization, March 19-20, 2016

National seminar on Defining Modern Biology: Plants and Microbes. Match 10, 2016.

INTERNATIONAL

2nd International conference on Bridging Innovations in Pharmaceutical, Medical and Biosciences, February 11-12, 2017.

2nd International conference on Frontier in Pharmaceutical Sciences and Research, 2017.

Appendix A

LIST OF CHEMICALS USED

CHEMICALS USED

A

ABTS

Acetic anhydride

Acetone

Aluminium chloride

Ammonia

Antimony chloride

Arnow's reagent

B

Benzene

Bismuth subnitrate

Butanol

C

Carbon tetrachloride

Carrageenan

Chloroform

Citrate buffer

Copper sulphate

D

Diethyl ether

DPPH

Dragendorff reagent

E

Ethanol

Ethyl acetate

F

Ferous chloride

Ferric chloride

Ferrozine

Folin-ciocalteau reagent

Folin-denis reagent

Formic acid

G

Glacial acetic acid

Glibenclamide

Glucose

Glycerine

Greiss reagent

H

HCl

Heptane

Hexane

Hippuryl-histidyl-leucine

I

Iodine

Isopropanol

K

KOH

L

Lead acetate

Liebermann burchard’s reagent

M

Methanol

MTT

NBT

N

Nicotinamide

Ninhydrin

Nitric acid

NP/PEG

P

Petroleum benzene

Petroleum ether

Phloroglucinol

Phospho molybdic acid

Phosphoric acid

PMS

PNPG

Potassium ferricyanide

Potassium hydroxide

Potassium iodide

Potassium persulphate

S

Silica

Sodium carbonate

Sodium hydroxide

Sodium molybdate

Sodium nitrite

Sodium nitroprusside

Sodium potassium tartarate

Sodium tungstate

Streptomycin

Streptozotocin

Sudan III

Sulphuric acid

Superoxide

T

Tannic acid

Toluene

Tri-chloro acetic acid (TCA)

V

Vanillin

Appendix B

ABBREVIATIONS USED

ABBREVIATION

A

AAAc	Acetone extract of formulation AA
AAAq	Aqueous extract of formulation AA
AABu	Butanol extract of formulation AA
AABz	Benzene extract for formulation AA
AACl	Cloroform extract for formulation AA
AAEa	Ethyl acetate extract for formulation AA
AAEt	Ethanol extract of formulation AA
AAHp	Heptane extract for formulation AA
AAHx	Hexane extract of formulation AA
AAMt	Methanol extract of formulation AA
AB	Autoclave boiling
ABTS	2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ACE	Angiotensin converting enzyme
AHA	Anti-hypertensive activity
ALP	Alkaline phosphatase
AR	Formulation for arthritis
ARAc	Acetone extract of formulation AR
ARAq	Aqueous extract of formulation AR
ARBu	Butanol extract of formulation AR
ARBz	Benzene extract for formulation AR
ARCl	Cloroform extract for formulation AR
AREa	Ethyl acetate extract for formulation AR
AREt	Ethanol extract of formulation AR
ARHp	Heptane extract for formulation AR
ARHx	Hexane extract of formulation AR
ARMt	Methanol extract of formulation AR
AS	Formulation for asthma
ASAc	Acetone extract of formulation AS
ASAq	Aqueous extract of formulation AS
ASBu	Butanol extract of formulation AS
ASBz	Benzene extract for formulation AS
ASCl	Cloroform extract for formulation AS
ASEa	Ethyl acetate extract for formulation AS
ASEt	Ethanolic extract of formulation AS
ASHp	Heptane extract for formulation AS
ASHx	Hexane extract of formulation AS
ASMt	Methanolic extract of formulation AS

B

BCG	Bromocresol green
BOFF	Bark of Frainus floribunda
BP	Formulation for High blood pressure
BPAc	Acetone extract of formulation BP
BPAq	Aqueous extract of formulation BP
BPBu	Butanol extract of formulation BP
BPBz	Benzene extract for formulation BP
BPCl	Cloroform extract for formulation BP
BPEa	Ethyl acetate extract for formulation BP
BPEt	Ethanol extract of formulation BP
BPHp	Heptane extract for formulation BP
BPHx	Hexane extract of formulation BP
BPMt	Methanol extract of formulation BP

C

CB	Formulation for sinusitis
CBAc	Acetone extract of formulation CB
CBAq	Aqueous extract of formulation CB
CBBu	Butanol extract of formulation CB
CBBz	Benzene extract for formulation CB
CBCl	Cloroform extract for formulation CB
CBEa	Ethyl acetate extract for formulation CB
CBEt	Ethanol extract of formulation CB
CBHp	Heptane extract for formulation CB
CBHx	Hexane extract of formulation CB
CBMt	Methanol extract of formulation CB
CE	Catechol equivalents
CONT	Control group
COX	Cycloxygenase
CP	Cold percolation

D

DMSO	Dimethyl Sufoixide
DPPH	1,1-diphenyl-2- picrylhydrazyl

E

EW	Extractive weight

F

FF	Formulation of diabetes
FFAc	Acetone extract of formulation FF
FFAq	Aqueous extract of formulation FF
FFBu	Butanol extract of formulation FF

FFBz	Benzene extract for formulation FF
FFCl	Cloroform extract for formulation FF
FFEa	Ethyl acetate extract for formulation FF
FFEt	Ethanol extract of formulation FF
FFHp	Heptane extract for formulation FF
FFHx	Hexane extract of formulation FF
FFMt	Methanol extract of formulation FF
FP	Formulation for food poisoning
FPAc	Acetone extract of formulation FP
FPAq	Aqueous extract of formulation FP
FPBu	Butanol extract of formulation FP
FPBz	Benzene extract for formulation FP
FPCl	Cloroform extract for formulation FP
FPEa	Ethyl acetate extract for formulation FP
FPEt	Ethanol extract of formulation FP
FPHp	Heptane extract for formulation FP
FPHx	Hexane extract of formulation FP
FPMt	Methanol extract of formulation FP
FRAP	Ferric reducing antioxidant power
FWT	Fresh weight tissue

G

GAE	Gallic acid equivalent
GC-MS	Gas Chromatography- Mass Spectroscopy
GS	formulation for gastritis
GSAc	Acetone extract of formulation GS
GSAq	Aqueous extract of formulation GS
GSBu	Butanol extract of formulation GS
GSBz	Benzene extract for formulation GS
GSCl	Cloroform extract for formulation GS
GSEa	Ethyl acetate extract for formulation GS
GSEt	Ethanol extract of formulation GS
GSHp	Heptane extract for formulation GS
GSHx	Hexane extract of formulation GS
GSK-3	Glycogen synthase kinase-3
GSMt	Methanol extract of formulation GS

H

HDL	High-density lipoprotein
HP	Formulation for heart palpitation
HPAc	Acetone extract of formulation HP
HPAq	Aqueous etxract of formulation HP
HPBu	Butanol etxract of formulation HP

HPBz	Benzene extract for formulation HP
HPCl	Cloroform extract for formulation HP
HPEa	Ethyl acetate extract for formulation HP
HPEt	Ethanol etxract of formulation HP
HPHp	Heptane extract for formulation HP
HPHx	Hexane extract of formulation HP
HPMt	Methanol extract of formulation HP
HPTLC	High Performance Thin Layer Chromatography
HS	Formulation for indigestion and stomachahe
HSBu	Butanol extract of formulation HS
HSBz	Benzene extract for formulation HS
HSCl	Cloroform extract for formulation HS
HSEa	Ethyl acetate extract for formulation HS
HSAc	Acetone extract of formulation HS
HSAq	Aqueous extract of formulation HS
HSEt	Ethanol extract of formulation HS
HSHp	Heptane extract for formulation HS
HSHx	Hexane extract of formulation HS
HSMt	Methanol extract of formulation HS

K

KOH	Potassium hydroxide

L

LDL	Low-density lipoprotein

M

MC	Metal chelation
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N

NADH	Nicotinamide adenine dinucleotide hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NB	Normal boiling
NIDDM	Non-insulin dependent diabetes mellitus
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
NO_2	Nitrogen dioxide
NOR	
CONT	Normal control group

O

OD	Optical density
OGTT	Oral Glucose Tolerance Test

P

PBS	Phosphate Buffer
PCA	Principal Component Analysis
pH	Power of hydrogen
PPHG	Postprandial hyperglycemia

Q

QE	Quercetin equivalent

R

RF	Retention factor
RNS	Radical Nitrogen Species
ROS	Reactive Oxygen Species

S

S	Soxhletion
SE	Standard error
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SO	Super oxide

T

TAC	Total alkaloid content
TAE	Tannic acid equivalents
TCL	Total Cholesterol
TFC	Total flavonoid content
TGL	Triglycerides
TLC	Thin Layer Chromatography
TOPC	Total orthodihydric phenol content
TPC	Total phenol content
TS	Formulation for tonsilitis
TSAc	Acetone extract of formulation TS
TSAq	Aqueous extract of formulation TS
TSBu	Butanol extract of formulation TS
TSBz	Benzene extract for formulation TS
TSC	Tannin Steroid content
TSCl	Cloroform extract for formulation TS
TSEa	Ethyl acetate extract for formulation TS
TSEt	Ethanol extract of formulation TS
TSHp	Heptane extract for formulation TS
TSHx	Hexane extract of formulation TS
TSMt	Methanol extract of formulation TS
TTC	Total Tannin content

Appendix C

LIST OF PUBLICATIONS FROM THE THESIS

LIST OF PUBLICATIONS

A Subba, P Mandal. 2015. Antioxidant potential of *Fraxinus floribunda* Bark Extracted through Various Aqueous Processing. *Free Radicals and Antioxidants*. 5: 6-12.

A Subba, P Mandal. 2015. Pharmacognostic studies and *in vitro* antioxidant potential of traditional polyherbal formulation of West Sikkim with *Asparagus* Spp. *Pharmacognosy Journal*. 7: 348-55.

A Subba, B Dutta, RK Sahu, P Mandal. 2017. Antioxidant, anti-inflammatory, and hepatoprotective activity of *Fraxinus floribunda* bark and the influence of extraction process on their bioactivity. 11: 983-90.

A Subba, P Mandal. 2019. Standardization of a traditional polyherbal formulation with pharmacognostic study; its phytochemical content, antioxidant, and antidiabetic activity. *Asian Journal of Pharmaceutical and Clinical Research*. 12: 182-90.

A Subba, RK Sahu, Bhardwaj S, P Mandal. 2019. Alpha glucosidase inhibiting activity and *in vivo* antidiabetic activity of *Fraxinus floribunda* bark in streptozotocin-induced diabetic rats. *Pharmacognosy Research*. 11: 273-78.

SEMINARS ATTENDED

NATIONAL

National conference of Plant Physiology, December 13-16, 2013.

National symposium on Recent Trends in Plant and Microbial Research, March 22-23, 2013.

National seminar on Plant and Microbe: Diversity and Utilization, March 19-20, 2016

National seminar on Defining Modern Biology: Plants and Microbes. Match 10, 2016.

INTERNATIONAL

2nd International conference on Bridging Innovations in Pharmaceutical, Medical and Biosciences, February 11-12, 2017.

2nd International conference on Frontier in Pharmaceutical Sciences and Research, 2017.

Appendix D

ANIMAL ETHICAL COMMITTEE CERTIFICATE

COLUMBIA INSTITUTE OF PHARMACY

Email : cipraipur@gmail.com, Visit us at : www.columbiaiop.ac.in

CIP/Admin/2017-18/ 242

Date: 09/01/2018

TO WHOMSOEVER IT MAY CONCERN

This is to certify that **Ms. Arunika Subba,** Research Scholar, Plant Physiology and Pharmacognosy Research Laboratory Department of Botany, University of North Bengal, Rajarammohanpur, Siliguri, West Bengal has carried out Antidiabetic activity of her project entitled **"Pharmacognostic Evaluation and *In Vitro* Antioxidant Potential of Some Ethnomedicines Used by Traditional Practitioners of West Sikkim"** in Pharmacology Department of the Institute. Her project has been approved by the Institutional Animal Ethical Committee of the Institute (1321/PO/ReBi/S/10/CPCSEA dated 22/10/2014). The experiment and the parameters assessed were universally accepted and done accordance with CPCSEA regulations.

Prof Amit Roy,
Principal

Principal
Columbia Institute of Pharmacy
RAIPUR (C.G.)

Appendix E

PUBLISHED ARTICLES

Antioxidant potential of *Fraxinus floribunda* Bark Extracted through Various Aqueous Processing

Subba Arunika, Mandal Palash*

Plant Physiology and Pharmacognosy Research Laboratory, Department of Botany, University of North Bengal, Siliguri-734013, West Bengal, INDIA.

ABSTRACT

Introduction: *Fraxinus floribunda* Wallich (Oleaceae) is an ethno-medicinal plant found in Sikkim, India. The leaves of the plant are traditionally used in fracture, dislocation and other inflammatory conditions. From our survey, it was found that the successive aqueous decoction of the bark of this plant is prescribed to diabetic patients by herbal practitioners. In the present study the bark of *Fraxinus floribunda* was extracted through various boiling procedures and cold percolation method to assess the effect of process variation on the antioxidant activity. **Methods:** The dried bark sample of *Fraxinus floribunda* was subjected to three hydrothermal procedures and cold percolation method. The obtained extracts were investigated spectrophotometrically against DPPH, $ABTS^+$, nitric oxide, superoxide and ferric reducing power. Metal chelating activity and anti-lipid peroxidation assay were also performed along with quantitative estimation of total phenolics, flavonols and ortho-dihydric phenol content. **Results:** The lowest IC_{50} values for DPPH (0.241 mg/ml), $ABTS^+$ (0.0284 mg/ml), nitric oxide (5.3151 mg/ml), anti-lipid peroxidation assay (2.29 mg/ml) were exhibited by the extract obtained through pressure boiling. The same sample also showed highest ferric reducing power and total phenol content. Maximum total flavonol and ortho-dihydric phenol content were exhibited by the extracts obtained through normal boiling and Soxhletion respectively. **Conclusion:** The bark of *Fraxinus floribunda* contains potentially active antioxidants. Extracts obtained through pressure boiling showed significant antioxidant activity therefore it seems to be the most effective method of extraction for acquiring antioxidants. The providing data can enrich the existing comprehensive data of antioxidant activity of plant materials.

Key words: Hydrothermal processing, Lipid peroxidation, Metal chelation, Percolation, Phenolics, superoxide,

INTRODUCTION

Fraxinus floribunda Wallich is a tree occurring in the Eastern Himalayas, Sikkim and Khasi hills. The leaves of the plant have been traditionally used for the treatment of fracture and dislocation.[1] To our knowledge, no studies are provided on the pharmacological properties of the bark of this plant. Hence the present study reveals free-radical scavenging activity and quantitative phytochemical screening on the bark of *F.floribunda* and their dynamic alteration during various thermal processing (boiling, pressure boiling and soxhletion) and cold percolation was also done to assess the effect of extraction process variation on antioxidant availability.

***Corresponding address:**
Dr. Palash Mandal
Plant Physiology and Pharmacognosy Research Laboratory, Department of Botany, University of North Bengal, Siliguri-734013, West Bengal, India.
E-mail: nbubotanypm@gmail.com

DOI: 10.5530/fra.2015.1.2

MATERIALS AND METHODS

Chemicals

Methanol, 2,2-diphenyl-1-picryl hydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide sodium salt monohydrate (NADH), sulfanilamide, glacial acetic acid, naphthyl ethylenediamine dihydrochloride (NED), potassium ferricyanide [$K_3Fe(CN)_6$], (TCA), thiobarbituric acid (TBA), trichloroacetic acid,$FeSO_4.7H_2O$, potassium hydroxide

(KOH),potassium dihydrogen orthophosphate (KH_2PO_4), ethylene-diamine tetra acetic acid (EDTA), 2-deoxyribose, ferric chloride ($FeCl_3$), ferrous chloride ($FeCl_2$), ferrozine, sodium nitroprusside, gallic acid, catechol,quercetin, Folin-Ciocalteau reagent, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), aluminium chloride (AlCl3), sodium hydroxide (NaOH), hydrochloric acid and potassium chloride. All these chemicals were either purchased from Himedia, India or of Merck, Germany made.

Plant material

The bark of *Fraxinus floribunda* Wallich was collected from Sankhu, Dentam, West Sikkim, India in the month of April, 2012. The specimen was identified and authenticated by Dr. A.P. Das, Professor, Taxonomy & Environmental Biology Laboratory, Department of Botany, University of North Bengal. A voucher specimen was deposited at laboratory herbarium, Taxonomy and Environmental Biology Laboratory, University of North Bengal and identified against the accession number 9632/ Tag no E.S.03 for the further references.

Animal material

For anti-lipid peroxidation assay, goat liver used was collected from slaughter house immediately after slay and the experiment was conducted within an hour after collection.

Sample preparation

The bark of *F. floribunda* was sun-dried & reduced to coarse powder using laboratory grinder. Powdered bark (10 g) was extracted with 100 ml distilled water separately through boiling at atmospheric pressure, soxhlet apparatus, autoclave and cold percolation at low temperature. The extracts were collected & filtered through Whatman No. 1 filter paper and finally subjected to evaporation in a heating mantle. Final volume make up of each of the sample were done by adding distilled water. This process was repeated three successive times to obtain the extracts at three different stages. The samples were then kept in bottles and stored in refrigerator for further analysis.

Extraction procedures

• *Soxhletion*

The dried bark powder (10 g) was extracted successively with 100 ml distilled water in a soxhlet extractor (Merck, Germany) for 8 hours.

• *Boiling at atmospheric pressure*

The conventional extraction was done through boiling / refluxing the sample for 2 hours in a conical flask.

• *High pressure boiling*

The powered sample was put into an autoclave for 15 mins under high pressure (1.5 kg cm^{-2}) and high temperature (121^oC).

• *Cold percolation*

The powdered sample was placed in a glass beaker with water at below 4^0C in a refrigerator for 48 hours. The sample was then filtered with Whatman no 1 filter paper.

Evaluation of antioxidant activity

Determination of the free radical scavenging activity of each of the crude extract was carried using the following methods.

• *DPPH radical scavenging activity*

DPPH based radical scavenging activity was measured.[2] Aqueous extract (0.2 ml) was added to 1.8 ml of DPPH (0.1 mM). The mixture was shaken well and allowed to stand in dark at room temperature for 10 minutes. The absorbance was measured at 517 nm with UV-Visible Spectrophotometer (Systronics, 2201). A reaction mixture without test sample was considered as control.DPPH scavenging activity (%) was measured using the following formula:

$$DPPH\ scavenging\ activity\ (\%) = \left[\frac{Absorbance_{control} - Absorbance_{sample}}{Absorbance_{control}}\right] \times 100$$

Where, absorbance of the control and absorbance of the extract or standard.

Then IC_{50} was calculated from the graph of percentage inhibitions plotted against concentration.

• *$ABTS^+$ radical scavenging assay*

$ABTS^+$ assay was done by the method[3] with few modifications. The reaction mixture contained 7mM $ABTS^+$ radical cation (s) in methanol with 2.4 mM potassium per sulfate ($K_2S_2O_8$) in water which was stored in the dark at room temperature for 12 hrs. Before usage, the $ABTS^+$ solution was diluted 8 times. Then, 2 ml of $ABTS^+$ solution was added to 1 ml of the aqueous extract. After 10 mins, absorbance at 734 nm was measured.

$$ABTS^+ scavenging\ effect\ (\%) = \left[\frac{Absorbance_{control} - Absorbance_{sample}}{Absorbance_{control}}\right] \times 100$$

• *Superoxide anion radical scavenging activity*

For superoxide radical scavenging activity method[4] was followed with slight modifications. The solutions were prepared in 0.1 M phosphate buffer (pH-7.4). The reaction mixture contained 1 ml of NBT solution (312 μM prepared

in phosphate buffer, pH-7.4), 1 ml of NADH solution (936 µM prepared in phosphate buffer, pH-7.4), and 1 ml of aqueous extract of different concentrations. After 5 minutes of incubation, 20 µl of PMS (120 µM) was added to the reaction mixture. The reactant was illuminated by fluorescent lamp for 30 minutes. The absorbance was measured at 560 nm against control. The percentage inhibition of generation of superoxide anion was calculated by using the following formula:

$$\text{Superoxide scavenging effect (\%)} = \left[\frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}}\right] \times 100$$

• *Metal chelating activity*

The method[5] was used for the estimation of chelating activity of the extracts for ferrous ions $Fe2^{+}$.To 0.4 ml of aqueous extract, 1.6 ml of water was diluted and mixed with 0.04 ml of $FeCl_2$ (2 mM). After 30 minutes, 0.08 ml ferrozine (5 mM) was added. The mixture was shaken vigorously and left standing at room temperature for 10 minutes. The absorbance of the solution was measured at 562 nm. The chelating activity was calculated as:

$$\text{Chelating effect (\%)} = \left[\frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}}\right] \times 100$$

• *Ferric reducing antioxidant power assay*

The ferric reducing antioxidant power (FRAP) can be determined.[6] The reaction mixture was prepared by mixing 1ml extract, 2.5 ml of phosphate buffer (200 mM, pH 6.6), 2.5 ml of potassium ferricyanide (30 mM) and the mixture was incubated at 50°C for 20 minutes. To the reaction mixture, 2.5 ml of trichloroacetic acid (600 mM was added and centrifuged for 10 minutes at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (6 mM) and absorbance was measured at 700 nm in UV-VIS spectrophotometer. Ascorbic acid was used as control.

• *Nitric oxide radical scavenging assay*

Nitric oxide was generated according to the method of Marcocci *et al*. (1994) from sodium nitroprusside and measured by the Greiss reaction.[7] The reaction mixture was prepared with 2 ml (15 mM) sodium nitroprusside, 0.5 ml PBS solution and 0.5 ml aqueous extract. After incubation at room temperature for 160 minutes, 3 ml Greiss reagent was mixed and again the mixture was left for incubation at 25°C for 30 minutes. Lastly absorbance was taken at 546 nm. Radical scavenging activity was expressed as percent inhibition from the given formula:

$$\text{Nitric oxide scavenging effect (\%)} = \left[\frac{\text{Absorbance}_{\text{cont}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{cont}}}\right] \times 100$$

• *Anti-lipid peroxidation assay*

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed using liver homogenates as lipid-rich media.[8] $FeSO_4$induces lipid peroxidation. Malondialdehyde (MDA), produced by oxidation of polyunsaturated fatty acids, reacted with two molecules of thiobarbituric acid (TBA) yielding a pinkish red chromogen with an absorbance maximum at 532 nm was measured. The inhibition percentage of lipid peroxidation by the extract at different concentration was calculated with the formula:

$$\text{Lipid peroxidation effect (\%)} = \left[\frac{A_0 - A_1}{A_0}\right] \times 100$$

Where, A_0 = absorbance before reaction and A_1 = absorbance after reaction has taken place.

Quantitative estimation of bioactive phytochemicals

• *Total phenol content*

The total phenolic content of the aqueous extract of *F. floribunda* was carried out with Folin-Ciocalteau reagent.[9] One ml of aqueous bark extract, 1 ml of 95% ethanol, 5 ml of distilled water, 0.5 ml 50% Folin-Ciocalteu reagent was mixed and after 5 mins 5% of sodium carbonate (Na_2CO_3) was added and after 1 hr the absorbance value was taken at 725 nm.

• *Total flavonol content*

Total flavonol content in the aqueous extract of *F. floribunda* was done by the method[10] Sodium nitrite (0.5 ml) was mixed to form the reaction mixture. After 5 mins 0.3 ml 10% aluminium chloride (AlCl3), was added and diluted with 2.5 ml of distilled water and mixed well. The absorbance was taken at 510 nm.

• *Total ortho-dihydric phenol content*

The determination of ortho-dihydric phenolics was based on the method.[11] Firstly; 0.5 ml of aqueous extract was added to a mixture containing 0.5 ml of 0.05 (N) HCl and 0.5 ml of Arnow's reagent. After proper shaking, 5 ml of water was added, followed by the addition of 2 ml of 1 (N) NaOH. Finally, after 5 min the absorbance was recorded at 515 nm. The total ortho-dihydric phenolics content in different extracts was calculated as catechol equivalent (CE)/gm fresh weight tissue (FWT).

• *Statistical analysis*

The standard software SPSS (ver. 15.0) was used for all statistical analysis. One-way analysis of variance (ANOVA) was used to compare the differences and the means which were considered significant at $p \leq 0.05$. Correlation and regression analysis was done by using Excel Software of MS Office 2009.

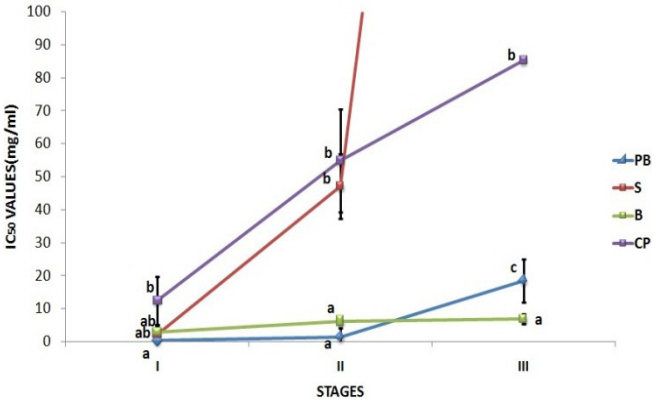


Figure 1: DPPH Scavenging Activity

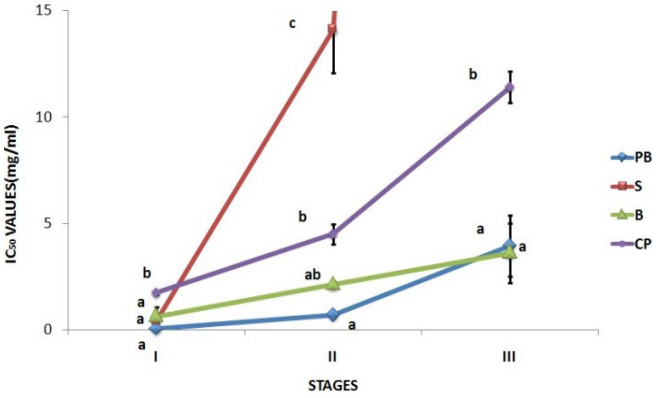


Figure 2: ABTS^{+} Scavenging Activity

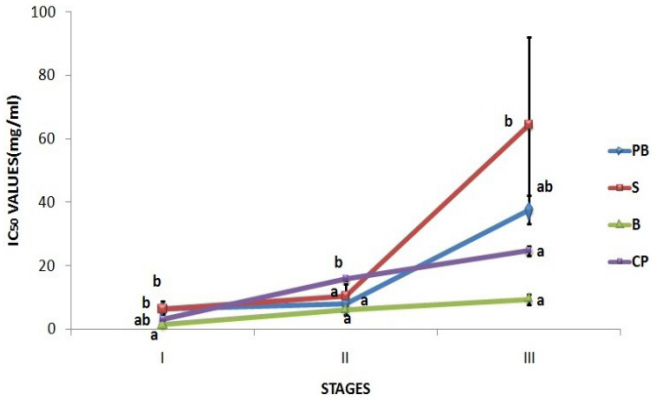


Figure 3: Superoxide Scavenging Activity

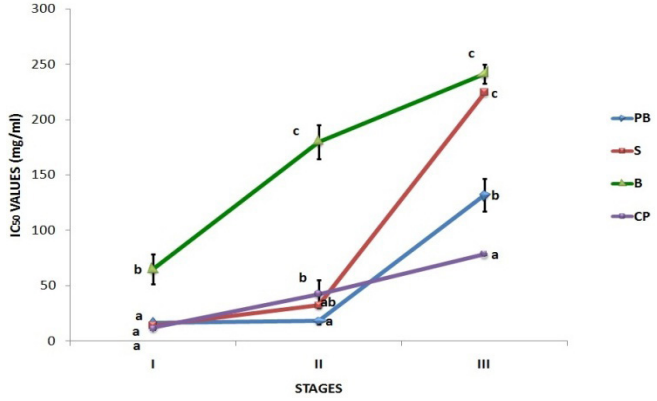


Figure 4: Metal Chelating Activity

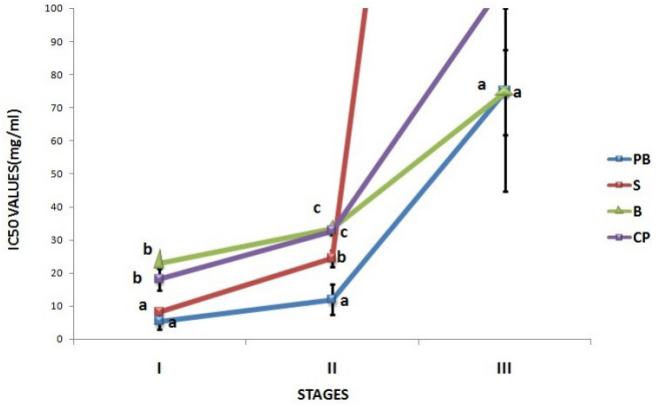


Figure 5: Nitric oxide Scavenging Activity

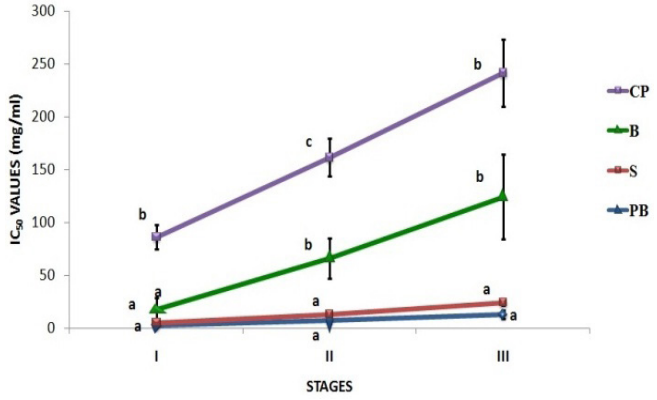


Figure 6: Antilipid Peroxidation Activity

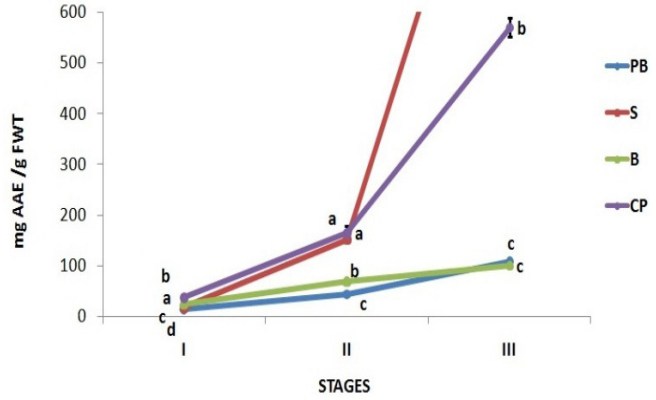


Figure 7: Ferric Reducing Antioxidant Potential

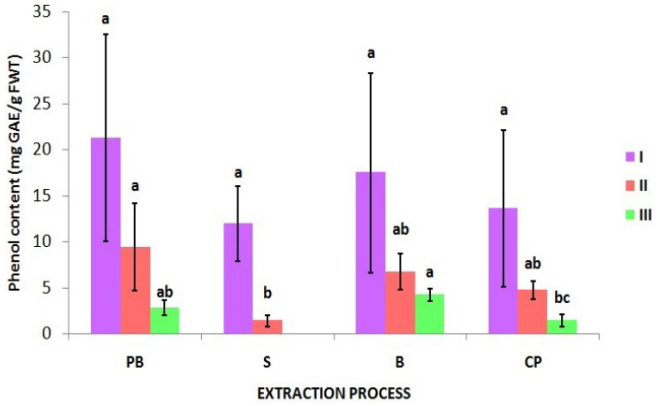


Figure 8: Total phenol content

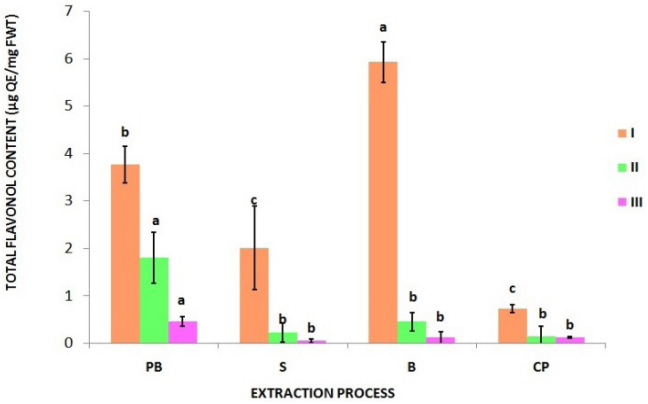


Figure 9: Total flavonol content

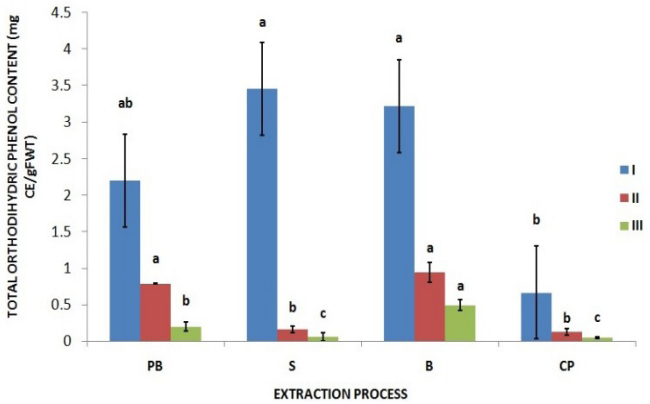


Figure 10: Total orthodihydric phenol content. I, II and III represents first, second and third successive stages of extraction. Values with different letters (a, b, c & d) are significantly ($p < 0.05$) different from each other by Duncan's multiple range test (DMRT).

RESULTS

From the results obtained, it might be stated that the bark of *F. floribunda* contains potentially active antioxidants. The radical scavenging activity of bark of *F. floribunda* obtained from different extraction procedures at three successive stages are presented in figure 1-6. In free radical scavenging assays such as DPPH (Figure 1), $ABTS^+$ (Figure 2), nitric oxide (Figure 5) and anti-lipid peroxidation activity (Figure 6), it was found that the extracts obtained from pressure boiling have shown lowest IC_{50} values in all cases as well as it has a good ferric oxide reducing potential (Figure 7) thus high antioxidant activity. But there are some cases where boiling has proved to be a better extraction method than other methods for superoxide scavenging activity (Figure 3) while cold percolation method has shown superior restoration of metal chelating capacity (Figure 4). Along with the free radical scavenging activities, some bioactive phytochemicals were also estimated such as total phenol content, total flavonol content and total ortho dihydric phenol content. The highest phenol content was exhibited by extracts from pressure boiling (Figure 8) while elevated flavonol content (Figure 9) was obtained by extracts from boiling and optimum (Figure 10) orthodihydric phenol content was obtained from extracts through soxhletion. Along with four different extraction methods, the bark was extracted in three successive stages in each method. This was done to observe the retention of antioxidant properties at different successive stages. From the graphs, it is clear that the antioxidant activity of the bark of the plant was retained till third stage except in soxhletion method as the antioxidant activity was negligible in the third stage. From the Pearson correlation test (Table 1), insignificant negative correlation was obtained between phenolics content and antioxidant activity assayed by different free radicals scavenging assays while significant positive correlation was observed between various radical scavenging assays such as $ABTS^+$, DPPH, superoxide and nitric oxide. Similarly phytochemical contents (phenol, flavonol and ortho dihydric phenol) were found to have significant positive correlation among them. Significant positive correlation was also observed between nitric oxide assay and metal chelation as well as between

Table 1: Correlations between the IC_{50} values of antioxidant activities, phenolics, flavonoids and orthodihydric phenol content of bark of *F. floribunda*

	DPPH	ABTS	TPC	TFC	TOPC	FRAP	SO	MC	NO
ABTS	0.985**	-	-	-	-	-	-	-	-
TPC	-0.431	-0.381	-	-	-	-	-	-	-
TFC	-0.301	-0.251	0.800**	-	-	-	-	-	-
TOPC	-0.226	-0.167	0.842**	0.674*	-	-	-	-	-
FRAP	0.279	0.132	-0.555	-0.432	-0.448	-	-		-
SO	0.870**	0.839**	-0.576	-0.431	-0.377	0.380	-	-	-
MC	0.481	0.510	-0.544	-0.375	-0.418	0.137	0.532	-	-
NO	0.975**	0.962**	-0.490	-0.331	-0.297	0.317	0.908**	0.629*	-
ALP	-0.095	-0.215	-0.378	-0.460	-0.528	0.595*	-0.071	0.248	-0.025

**.Correlation is significant at the 0.01 level (2-tailed).

*Correlation is significant at the 0.05 level (2-tailed).

DPPH1: 1-diphenyl-2-picrylhydrazyl; ABTS: 2,2´-azinobis-3-ethylbenzo-thiozoline-6-sulphonic acid; TPC: Total phenol content; TFC: Total flavonol content; TOPC: Total orthodihydric phenol content; FRAP: Ferric reducing antioxidant power; SO: Superoxide; MC: Metal chelation; NO: Nitric oxide; ALP: Anti-lipid peroxidation.

anti-lipid peroxidation and reducing power.

DISCUSSION

Plants are the main source of natural antioxidants, which are greatly important for human health.[12] Several natural antioxidants have already been isolated from various plant materials such as leafy vegetables, fruits, seeds, cereals[13] and had showed ROS (reactive oxygen species) scavenging activity and prevention of lipid peroxidation.[14,15] The decoction of aqueous extracts of fruits of Dilleniaindica used by the Assamese people in their cuisine was found to be an ideal source of antioxidants.[16] This protection could have been acquired due to the presence of antioxidant molecules in plants and the products of secondary metabolites such as phenolics, flavonoids and polypropanoids which have the capacity to scavenge free radicals due to their ability to donate proton.

Extraction procedure is one of the crucial points for the extraction of various bioactive compounds. The influence of variation in extraction solvents and techniques on the content of natural antioxidants in extracts has been reported by many researchers.[17,18] In the present study, the bark of *Fraxinus floribunda* has been extracted through four different methods including normal/conventional boiling, pressure boiling by using autoclave, soxhlet extraction and cold percolation. From the results, it was revealed that the bark of *F. floribunda* contains potentially active antioxidants and free radical scavenging activity. It was observed that the sample extracted through pressure boiling has exhibited highest free radical scavenging activity, reducing antioxidant power as well as highest total phenol content. Similar result was obtained in case of ginseng seeds where total amount of phenolic compounds were about three times higher when processed under high pressure through autoclaving at 1300 C than in seeds that were not treated.[19] Autoclaving for a longer time enhanced the diffusion of phenols from seed coats to cooking water and from there to cotyledons which caused increased levels of all tocopherols and tocotrienols along with the increase in vanillin and ferulic and p-coumaric acids.[20] But in case of total flavonol content and superoxide radical scavenging assay, the sample extracted through normal boiling have showed the best result. Soxhletion has proved to be a good extraction procedure for the isolation of total ortho dihydric phenol content while cold percolation method has showed moderate result in all the assays.

A statistically insignificant negative correlation was obtained between phenolic contents and various free radical scavenging activities. Hence this data indicated that the phenolic compounds might not be contributing for the high antioxidant activities in the aqueous bark extract of *F. floribunda*. Similar result was obtained in the aqueous extract of Brazilian mushroom and button mushroom as their aqueous extract showed no correlation between the phenolic compounds and antioxidant potential.[21] Thus, it is reasonable to state that phenolics may not act as the most important antioxidant components in the aqueous extracts of bark of *Fraxinus floribunda*. There might be some active non-phenolic constituents that can be extracted in aqueous system. The presence of non-phenolic compounds like uronic acids and amino acids might also be suggested that can produce higher antioxidant capacity than those generated by polyphenols.[22,23]

CONCLUSION

The purpose of the present experiment was to compare changes in phytochemical content and antioxidant activities from the bark of *Fraxinus floribunda* and measuring alteration of bioactivity of the samples extracted through process variation (boiling at atmospheric pressure, pressure boiling, soxhlet decoction and cold percolation). Results indicated that various extraction methods have significant influence on the antioxidant property as well as phytochemical content of extracts. Based on the above presented results, various extraction methods might be applied to isolate pharmacologically active components from *F. floribunda*, especially the extracts obtained through pressure boiling exhibited the most significant antioxidant activities, and therefore it seemed to be the more efficacious method for acquiring antioxidants. The results and findings of this study support this view that the bark of *Fraxinus floribunda* is a promising source natural antioxidant which might help in preventing the progress of various oxidative stress mediated disorders.

ACKNOWLEDGEMENT

The authors are grateful to the University Grants Commission (UGC), India for providing Financial support for the research under Rajiv Gandhi National Fellowship scheme.

REFERENCES

1. Gurung B. The medicinal plants of the Sikkim Himalaya, Subash publication, Gangtok, Sikkim; 2002.
2. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 1958; 181: 1199-200.
3. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 1999; 26(9-10): 1231-7.
4. Nishikimi M, Appaji N, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazinemethosulfate and molecular oxygen. Biochem Biophys Res Commun. 1972; 46(2): 849-54.
5. Dinis TC, Madeira VM, Almeida LM. Action of phenolic derivates (acetoaminophen, salycilate, and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch Bio chem Biophys. 1994; 315(1): 161-9.
6. Oyaizu M. Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucoamine. Jpn J Nutr. 1986; 4(6): 307-15.
7. Marcocci L, Packer L, Droy-Lefaix MT, Sekaki A, Gardes-Albert M. Antioxidant action of *Ginkgo biloba* extract EGb 761. Methods Enzymol 1994; 234: 462-75.
8. Bouchet N, Barrier L, Famconneau B. Radical scavenging activity and antioxidant properties of tannins from *Guiera senegalensis* (Combretaceae). Phytother Res. 1998; 12(3): 159-62.
9. Chandler SF, Dodds JH. The effect of phosphate, nitrogen and sucrose on the production of phenolics and solasidine in callus cultures of *Solanum laciniatum*. Plant Cell Rep. 1983; 2(4): 205–8.
10. Sultana B, Anwar F, Ashraf M. Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. Molecules 2009; 14(6): 2167-80.
11. Kim DO, Jeong SW, Lee CY. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. Food Chem. 2003; 81(3): 321- 6.
12. Parasuraman S, Thing GS, Dhanaraj SA. Polyherbal formulation: Concept of ayurveda. Phcog Rev. 2014; 8(16): 73-80.
13. Pokorny J. Natural antioxidants for food use. Trends Food Sci Technol. 1991; 2: 223-7.
14. Parasuraman S, Kumar E, Kumar A, Emerson S. Free radical scavenging property and diuretic effect of triglize, a polyherbal formulation in experimental models. J Pharmacol Pharmacother. 2010; 1(1): 38-41.
15. Aqil F, Ahmad I, Mehmoud Z. Antioxidant and free radical scavenging properties of twelve traditionally used Indian medicinal plants. Turk J Biol. 2006; 30: 177-83.
16. Das M, Sarma BP, Ahmed G, Nirmala CB, Choudhury MK. In vitro antioxidant activity and total phenolic content of *Dillenia indica* and *Garcinia penducalata*, commonly used fruits in Assamese cuisine. Free Rad Antiox. 2012; 2(2): 30-6.
17. Grigonisa D, Venskutonis PR, Sivik B, Sandahl M, Eskilsson CS. Comparison of different extraction techniques for isolation of antioxidants from sweet grass (*Hierochl oeodorata*). Journal Super crit Fluids 2005; 33(3): 223-33.
18. Michiels JA, Kevers C, Pincemail J, Defraigne JO, Dommes J. Extraction conditions can greatly influence antioxidant capacity assays in plant food matrices. Food Chem. 2012; 130(4): 986-93.
19. Bae HM, Kim SS, Cho CW, Yang DC, Ko SK, Kim KT. Antioxidant activities of ginseng seeds treated by autoclaving. J Ginseng Res. 2012; 36(4): 411-7.
20. Bryngelsson S, Dimberg LH, Kamal-Eldin A. Effects of commercial processing on levels of antioxidants in oats (*Avena sativa L*.). J Agric Food Chem. 2002; 50(7): 1890-96.
21. Gan CH, Nurul Amira B, Asmah R. Antioxidant analysis of different types of edible mushrooms (*Agaricus bisporous* and *Agaricus brasiliensis*). Int Food Res J. 2013; 20(3): 1095-2.
22. Chye FY, Wong JY, Lee JS. Nutritional quality and antioxidant activity of selected edible wild mushrooms. Food Sci Technol Int. 2008; 14(4): 375-84.
23. Perez-Jimenez J, Saura-Calixto F. Antioxidant capacity of dietary polyphenols determined by ABTS assay: a kinetic expression of the results. Int J Food Sci Technol. 2006; 43(1): 185-91.

Pharmacogn. J.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcogfirst.com/phcogj

Original Article

Pharmacognostic Studies and *In Vitro* Antioxidant Potential of Traditional Polyherbal Formulation of West Sikkim with *Asparagus* Spp

Arunika Subba and Palash Mandal*

Department of Botany, Plant Physiology and Pharmacognosy Research Laboratory, University of North Bengal, Siliguri, West Bengal, India.

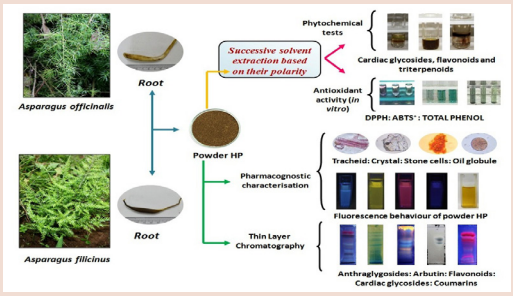
ABSTRACT

Introduction: The powder mixture of the two species of *Asparagaceae* (*Asparagus filicinus* and *Asparagus officinalis*) was found to be used traditionally for the treatment of heart palpitation in west Sikkim. **Objective:** Pharmacognostic characterisation was carried out for the authentication of the powder drug which included powder microscopy, fluorescence analysis and physicochemical characterisation. The presence of any therapeutic potential in HP was also determined by qualitative and quantitative estimation of phytochemicals along with free radical scavenging activity of various successive solvent extracts (based on their polarity). Thin layer chromatography (TLC) of the powdered HP was also done. The standard software SPSS (ver. 15.0) and XLSTAT 2009 (Addinsoft) and Smith's Statistical Package were used for different statistical analysis. **Results:** Powder microscopy of HP revealed the presence of calcium oxalate crystal, tracheids, stone cells etc. Various fluorescence colours were exhibited by HP on UV after reacting with different chemical reagents. The analysis values were also obtained in a satisfactory way. TLC and qualitative phytochemical analysis revealed the presence of some active phytoconstituents. Among all the solvent extracts, acetone, heptane, ethyl acetate and benzene extracts showed higher antioxidant potential. **Conclusion:** The results support the use of HP as a traditional medicine and further purification should be done for the identification of bioactive phytoconstituents responsible for its antioxidant activity.

Key words: Antioxidant, Pharmacognostic evaluation, Phytoconstituents, Successive solvent extraction, Thin layer Chromatography.

SUMMARY

- HP powder prepared from the roots of *Asparagus officinalis* and *Asparagus filicinus* is used to cure heart palpitation in the traditional system at a village in west Sikkim.
- Powder was subjected for some pharamacognostic characterisation methods and the result of which might be helpful in authentication of the traditional drug.
- Some phytoconstituents such as anthraglycosides, arbutin, cardiac glycosides, flavonoids, bitter principles and coumarins were revealed on the sample by separating through thin layer chromatography. Alkaloids and saponins were absent.
- Qualitative evaluation of phytochemicals revealed presence of cardiac glycosides and flavonoids in all solvent extracts while amino acid and triterpenoids in few extracts only and showed absence of tannin, resin and alkaloids.
- Antioxidants potential varied in different solvent extracts but overall acetone and heptane extracts were better than other extracts.



PICTORIAL ABSTRACT

Abbreviations used: HP: powder polyherb for heart palpitation, HPHx: Hexane extract, HPHp: Heptane extract, HPBz: Benzene extract, HPEa: Ethyl acetate extract, HPCl: Chloroform extract, HPAc: Acetone extract, HPBu: Butanol extract, HPEt: Ethanol extract, HPMt: Methanol extract, HPAq: Aqueous extract, TLC: Thin layer chromatography, DPPH: 1, 1-diphenyl-2-picrylhydrazyl scavenging activity, ABTS+: 2,2'-azinobis-3-ethylbenzothiozoline-6-sulphonic acid scavenging activity, SO: Superoxide scavenging activity, NO: Nitric oxide scavenging activity, MC: Metal chelating activity, FRAP: Ferric reducing antioxidant power, TPC: Total phenol content, TFC: Total flavonol content, TOPC: Total orthodihydric phenol content, GAE/mg EW: Gallic acid equivalent/mg extractive weight, QE/mg EW: quercetin equivalent/mg extractive weight, AAE/mg EW: Ascorbic acid equivalent/mg extractive weight.

Correspondence:

Dr. Palash Mandal, Department of Botany, Plant Physiology and Pharmacognosy Research Laboratory, University of North Bengal, Siliguri, West Bengal, P.O. 734 013, India.

Phone no: +91 9434886123

Email: nbubotanypm@gmail.com
DOI : 10.5530/pj.2015.6.6

INTRODUCTION

Since ancient times, plants have been used as herbal medicines to heal various ailments. Recently, there is widespread interest growing on drugs derived from plants because they are reliable, safe and cost effective compared with expensive synthetic drugs causing adverse effects in human health. Most of the noble drugs used in modern medicines are produced indirectly from medicinal plants[1] and about 90% of raw materials come from wild sources. But ethnomedicinal knowledge has still now confined to certain areas or group of people due to the lack of effective scientific communication.[2] Hence it is quite essential to establish the potential of herbal medicine and encourage the study of folk knowledge about medicinal plants.[3] In fact pharmaceutical and clinical investigations have raised the position of medicinal plants by identifying the active principle components present in them and elaborating their role in human and animal systems.[4]

Sikkim is a small Himalayan state (7096 km^2 geographical area) in Northeast region of India harbouring an extremely rich diversity of medicinal plants. Over the ages, Sikkim has developed rich cultural practices of folk medicines.[5] *Asparagus officinalis* Linn. and *Asparagus filicinus* Buchanon-Hamilton ex. D.Don., both belonging to family *Asparagaceae* are commonly used ethnomedicinal plants. Young stems of *A. officinalis* are used for the treatment of constipation and intestinal disorders.[6] Tubers of *A. filicinus* is traditionally used for the treatment of diarrhoea, dysentery,

diabetes[7] and it was also found to have antiviral activity against Influenza virus A.[8] Our survey revealed that the polyherbal formulation (HP) is made by mixing the roots of both the plants by an herbal practitioner for the treatment of heart palpitation. However the medicine prepared by traditional methods may not have the desired quality and batch to batch consistency. Hence this formulation required standardization with scientific parameters including organoleptic characters, physicochemical analysis and microscopy.

Recently the role of medicinal plants in disease control or prevention has been credited to antioxidant properties of their constituents usually polyphenolic compounds.[9] The intake of natural antioxidants are related to minimizing the risks of common but hazardous diseases like cancer, diabetes, cardiovascular problems, and many other diseases related with ageing.[10,11] Natural antioxidants have been observed in various unexplored plant resources like wild fruits,[12] leafy vegetables[13] and even bryophytes.[14]

Extraction of antioxidants from a plant material can be done by using different techniques and different solvents as these compounds have diverse chemical nature and often unique distribution in the plant matrix.[15,16] Solvent extraction is the most commonly used method for recovery of the plant antioxidants; however, the yields and antioxidant value of the plant extracts are strongly dependent on the polarity of the solvent plus the chemical nature of the isolated compounds.[17]

Literature survey did not yield any information about pharmacognostic studies of the above polyherb. There were no records provided on the antioxidant activity also. Therefore this study was focused on the standardization of HP, which was made from the roots of *A. officinalis* and *A. filicinus*, by measuring various attributes such as powder microscopy, fluorescence analysis and physicochemical studies. To explore the presence of active phytoconstituents, qualitative and quantitative phytochemicals content were also done along with free radicals scavenging activities of HP extracted in different solvents.

MATERIALS AND METHODS

Collection and authentication of plant material

A polyherbal powder sample prepared from the roots of *A. officinalis* and *A. filicinus* in equal ratio was collected from an herbal practitioner from Uttarey, West Sikkim in April, 2014. The herbarium specimen was identified and authenticated by Dr. A.P. Das, Professor, Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal. The voucher specimens were deposited at laboratory herbarium, Taxonomy and Environmental Biology Laboratory, University of North Bengal against accession no. 09724/Tag no. E.S. 05 and 09723/Tag no. E.S. 04 for *Asparagus filicinus* Buchanon-Hamilton ex. D.Don., and *Asparagus officinalis* Linn. respectively.

Sample preparation

The powder sample was extracted through soxhlet apparatus in 10 different solvents based on their polarity. The solvent extracts were hexane (HPHx), heptane (HPHp), benzene (HPBz), ethyl acetate (HPEa), chloroform (HPCl), acetone (HPAc), butanol (HPBu), ethanol (HPEt), methanol (HPMt) and water (HPAq). The samples were then evaporated and reconstituted in methanol. These extracted samples were used for qualitative and quantitative estimation of phytochemicals and determination of free radical scavenging activities. For TLC and pharmacognostic studies, the sample was used in its powder form.

Pharmacognostic characterization

Organoleptic evaluation and Powder microscopy

The colour, odour, taste and texture of the powder of HP were observed and recorded.[18] Microscopic examination was carried out in a standard method.[19] A small amount of powder of HP was taken on glass slide and mounted on glycerine and observed under microscope. For the observation of lignified tissues, powder was stained with alcoholic solution of phloroglucinol followed by concentrated HCl. Similarly the powder was also stained with N/10 iodine solution to observe the starch granules and for the identification of fixed oil and fats, Sudan III were used.

Fluorescence behaviour

The powder sample as such and after treatment with various chemical reagents was subjected to fluorescence analysis. Observations were made under visible light and under UV light of short (254 nm) and long wave length (365 nm) separately.[20]

Physico-chemical parameters

Coarse powder of the plant root was used to perform quality control parameters such as total ash, acid insoluble and water soluble ash, water and alcohol soluble extractive values and loss on drying.[21] Three determinations were carried out for each parameter.

Preliminary photochemical studies

The HP powder extracted in various solvents were subjected for preliminary phytochemical screening to observe the presence or absence of phytoconstituents like tannin, triterpeniods, amino acid, resin, cardiac glycosides and flavonoids by the standard methods.[22-25]

Thin layer chromatography

TLC was performed to analyse the variation in bioactive chemical constituents.[26] Readymade TLC plates (coated with silica gel 60 F_{254} on aluminium sheets) purchased from Merck Germany were used. The powdered sample was extracted with different procedures for the identification of each of the active constituents i.e. anthraglycosides, arbutin, cardiac glycosides, flavonoids, bitter principles, saponins, coumarins and alkaloids. The mobile phase solvent systems used were ethyl acetate: methanol: water (100:13.5:10) for the detection of anthraglycosides, arbutin, cardiac glycosides, bitter principles and alkaloids. The mobile phase ethyl acetate : formic acid : glacial acetic acid : water (100:11:11:26) was used for flavonoids identification and for the identification of saponin, the solvent system of chloroform: glacial acetic acid : methanol: water (64:32:12:8) was used while for the identification of coumarins, toluene : ethyl acetate (93:7) was used. The developed chromatograms were analyzed for presence of drug constituents by spraying with an appropriate group reagent/s. The chromatograms were then observed under UV-254 nm and UV-365 nm light. Photos were taken with DSLR Nikon camera (D-3200) and the R_f values were calculated with the following formula.

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Quantitative evaluation of phytochemicals

Total phenol content (TPC)

Folin-Ciocalteu reagent was used to determine the TPC of HP.[27] The absorbance was measured at 725 nm in UV-visible spectrophotometer. Total phenol values were expressed in terms of Gallic acid equivalent (GAE)/mg Extractive weight (EW).

Total flavonol content (TFC)

TFC was measured with the mixture consisted of methanolic extract with Sodium nitrite ($NaNO_2$), Aluminium chloride ($AlCl_3$) and Sodium hydroxide (NaOH).[17] The absorbance was measured at 510 nm. TFC in different extracts was calculated as quercetin equivalent (QE)/mg EW.

Total ortho-dihydric phenol content (TOPC)

TOPC was estimated according to standard method by using Arnow's reagent.[28] The OD values were taken at 515 nm. TOPC was calculated from a catechol standard curve.

Evaluation of antioxidants

DPPH radical scavenging activity

The free radical scavenging activity of all the solvent fractions of HP was evaluated using DPPH.[29] Discoloration of these extracts was measured at 517 nm after incubation for 10 minutes in dark at room temperature. Percentage of inhibition was calculated with the following formula:

$$\frac{I=A_0-A_1}{A_0} \times 100 \qquad \text{Equation (1)}$$

where A_0=absorbance of the control, A_1=absorbance of the sample (different fractions of HP), I=percent inhibition of DPPH by the sample.

ABTS⁺ scavenging activity

The ABTS⁺ free radical scavenging activity of all the solvent fractions of HP was done.[30] The diluted ABTS⁺ solution (2 ml) was added to 1 ml methanolic sample. The scavenging activity of sample was observed and absorbance was read at 734 nm after 10 minutes of incubation. The percentage inhibition was calculated by using Equation (1).

Superoxide scavenging assay (SO)

A standard method was used with some slight modifications to carry out SO scavenging activity.[31] Superoxide anions are produced in the PMS/NADH-NBT system. The decrease in absorbance shows the radical scavenging activity of the sample which was measured at 560 nm. The inhibition percentage was calculated by Equation (1).

Inhibition of nitric oxide production (NO)

At a physiological pH, aqueous sodium nitroprusside generates nitric oxide (NO) which produces nitrite after interacting with oxygen which can be estimated by Greiss reagent.[32] The diazotization of nitrite with sulphanilamide following its coupling with naphthylethylenediamine forms chromophore, the absorbance of which was measured at 546 nm. Equation (1) was used to calculate the percentage inhibition.

Metal chelating assay (MC)

The chelation of ferrous ions by HP extracts was estimated.[33] The colour complex formed by the chelation of Fe^{2+} by ferrozine was reduced by the chelating activity of sample competing with ferrozine was measured at 562 nm. The inhibition percentage was calculated using Equation (1).

Ferric reducing antioxidant power (FRAP)

The FRAP of the HP extracts was determined on the ability of the sample to reduce ferric ions.[34] Absorbance was measured in UV-VIS spectrophotometer at 700 nm. Ascorbic acid was taken as standard.

Statistical analysis

The standard software SPSS (ver. 15.0) was used for different statistical analysis. One-way analysis of variance (ANOVA) was used to compare the differences and the means which were considered significant at $p \leq 0.05$. Correlation and regression analysis were done by using XLSTAT 2009 (Addinsoft) and Smith's Statistical Package.

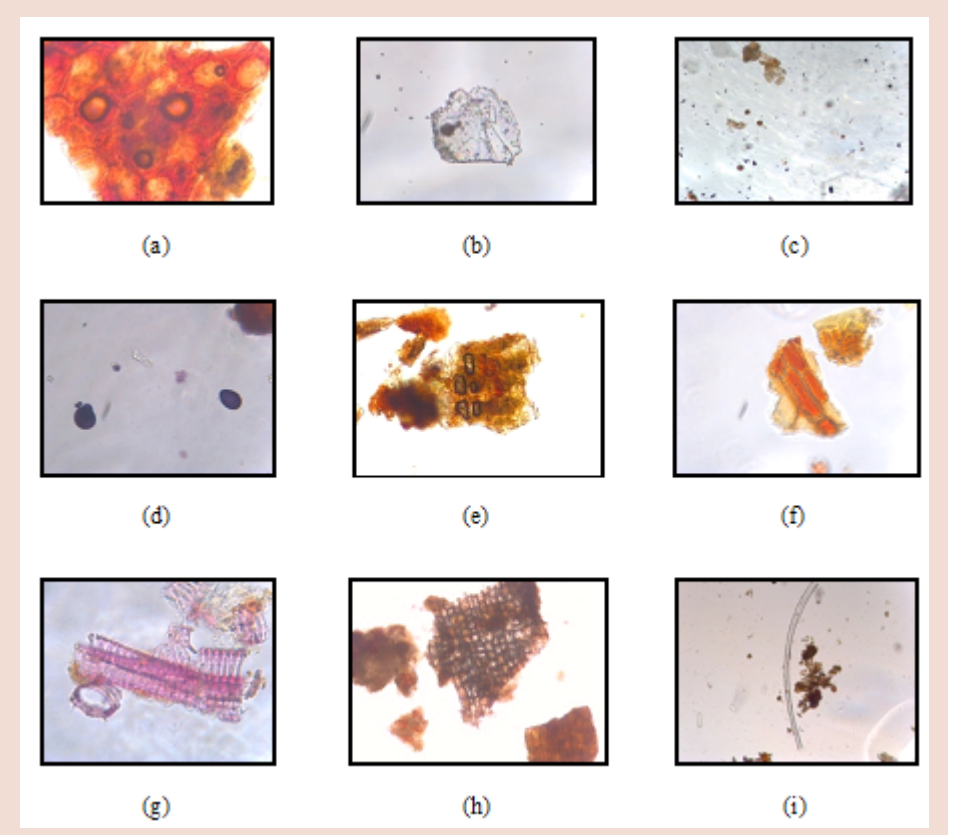


Figure 1: Powder microscopy of polyherb, HP

a) Collenchyma b) Prismatic calcium oxalate crystal c) Oil globules d) Starch e) Stone cells f) Parenchymatous cells g) Scalariform tracheid h) Cork cells i) Fiber

Table 1: Fluorescence analysis of powder HP

Reagents	Visible	UV-254	UV-365
Powder + distilled water	Green beige	Azure blue	Brilliant blue
Powder + 50% KOH	Red orange	Colourless	Black green
Powder + benzene	Sulphur yellow	Curry yellow	Broom yellow
Powder + 50% benzene	Fern green	Colourless	Raspberry red
Powder + chloroform	Colourless	Colourless	Distant blue
Powder + 50% chloroform	Fern green	Pearl blackberry	Rose
Powder + nitric acid (conc.)	Broom yellow	Colourless	Colourless
Powder + 50% nitric acid	Broom yellow	Colourless	Colourless
Powder + 10% ferric chloride	Broom yellow	Colourless	Colourless
Powder + methanol	Colourless	Cobalt blue	Ultramine blue
Powder + 50% methanol	Khaki grey	Grey blue	Gentian blue
Powder + ethanol	Colourless	Cobalt blue	Ultramine blue
Powder + 50% ethanol	Olive grey	Pearl night blue	Traffic blue
Powder + glacial acetic acid (conc.)	Reed green	Yellow olive	Brown beige
Powder + 50% glacial acetic acid	Green beige	No colour	Ultramine blue
Powder + sulphuric acid (conc.)	Ochre brown	Colourless	Blue grey
Powder + 50% sulphuric acid	Ochre yellow	Colourless	Grey blue

RESULTS

Organoleptic and powder microscopic evaluation

The powder of HP was brown in colour, slightly rough in texture and slightly bitter in taste. It had a characteristic smell. Powder microscopy revealed calcium oxalate crystal, parenchymatous cells, fiber, collenchyma, stone cells, cork cells and scalariform tracheid (Figure 1).

Fluorescence analysis

The powder of HP exhibited different colours after treating with different chemical reagents. The colours were observed in daylight and under ultraviolet (UV) light at 254 nm and 365 nm. The colours were identified and noted down in Table 1 using the standard colour chart of RAL.

Physicochemical characteristics

The results obtained from physicochemical studies were given in Table 2. The result of total ash (20.24%) showed that the powder HP contains both organic and inorganic matter. Total ash is mainly essential to check the purity of drugs showing the presence or absence of metallic salts or silica[35,36] which was found to be 20% w/w. The percentage of ash was calculated with reference to the air-dried powder. The acid insoluble ash was noted as 16% w/w while the presence of material exhausted by water i.e. the water soluble ash was recorded to be 14% w/w. Determination of extractive values is another way of checking the purity of the herbal formulation. The water soluble extractive value was found to be 3.21% w/w which signifies that more amount of constituents in HP was soluble in water than in alcohol (0.441% w/w). The percentage of loss on drying i.e. the moisture content in the powder was found to be 10.52% w/w.

Table 2: Physicochemical analysis of powder of HP

Parameter	Value
Total Ash(%) w/w	20.243
Acid insoluble ash(%) w/w	16.011
Water soluble ash(%) w/w	14.01
Alcohol soluble extractive value(%) w/w	0.441
Water soluble extractive value(%) w/w	3.211
Loss on drying(%) w/w	10.52
pH (1% w/v)	6.79
pH (10% w/v)	6.38

Thin layer chromatography

Preliminary investigation of active constituents in plants was carried out by thin layer chromatography (TLC) technique. After applying specific spraying reagents for a particular active constituent, the results of TLC showed the presence of anthraglycosides, arbutin, flavonoids, cardiac glycosides and coumarins in HP. R_f values were calculated for all the spots and the results of this analysis are described in Table 3.

Qualitative phytochemical analysis

The pharmacological activity of the crude drug mainly depends on the metabolites present in it. Thus preliminary screening of phytochemicals which was performed to establish a chemical profile of a crude drug was a part of chemical evaluation.[37,38] These phytochemical tests (Table 4) revealed the presence of cardiac glycosides and flavonoids in different extracts of HP obtained by using different solvents. Tannins, alkaloids and resins were absent while amino acid was found to be present in aqueous extract only and triterpenoids were found to be present in few extracts (acetone, butanol, ethanol, methanol and water).

Antioxidant activity

In the present study, various methods such as DPPH, $ABTS^+$, SO, NO, MC, FRAP were used to evaluate the antioxidant potential of HP. The sample was prepared in a soxhlet apparatus successively in various solvents (non-polar to polar). TPC, TFC and TOPC were also estimated quantitatively for HP (Table 5). The highest TPC was exhibited by heptane extract (361.41 mg GAE/mg EW) followed by acetone (272.99 mg GAE/mg EW). The highest TFC content (15.45 mg GAE/mg EW) was shown by benzene extract. For the radical scavenging activity, it is very clear from Table 5 that the acetone extract of HP showed the lowest IC_{50} values for DPPH, $ABTS^+$ and superoxide scavenging assays along with highest ferric reducing power indicating the highest antioxidant activity. The same extract showed excellent quantity of TPC and TFC also.

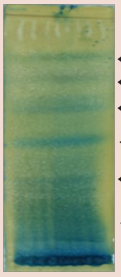
DISCUSSION

Traditional medicine has always played an important role in primary health care. But for the proper utilization of this medicine, each and every formulation mentioned in our indigenous knowledge of medicine should be scientifically evaluated.[39] Therefore the powder of HP was subjected to certain standardization parameters. Despite of the availability of various modern tools for the evaluation of plant drugs, powder microscopy is still considered as the simplest and cheapest method for identification of the source materials.[40] When powder of HP was subjected to microscopic analysis, starch granules were revealed after staining with iodine solution while Sudan III stain showed the presence of

Table 3: Detection of active phytoconstituents in powder HP by TLC method

Ethyl acetate: methanol: water (100:13.5:10)

Red band=Anthraglycosides

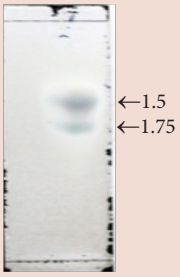


Blue band=Arbutin

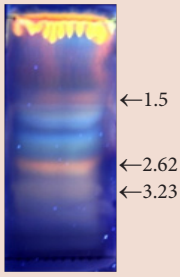
Ethyl acetate: Formic acid: glacial acetic acid: water (100:11:11:26)

Red band=Bitter Principles

Toluene: Ethyl acetate (93:7)

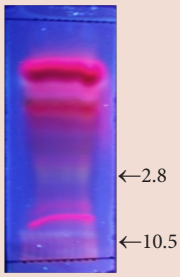


Blue band=Cardiac Glycosides



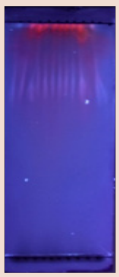
Yellow to orange bands=Flavonoids

Chloroform: Glacial acetic acid: Methanol: Water (64:32:12:8)



Blue band=Coumarin

Ethyl acetate: methanol: water (100:13.5:10)



Violet band=Saponins (Absent)

Brown band= alkaloids (Absent)

oil globules in the powder. Lignified tissues appeared pink in colour distributed abundantly after staining with phloroglucinol solution. Fluorescence study is also an important parameter for standardization of crude drug. Various chemical constituents present in plant materials exhibited fluorescence when suitably illuminated. Fluorescence colour of each compound is very specific. But if those chemicals itself are not fluorescent then they may be treated with different chemical reagents to attain fluorescence.[41] The quality and purity of crude drugs can also be checked by ash values. It indicates the presence of various impurities in crude drugs due to adulteration or incorrect processing during drying or storage or formulation. The water soluble ash shows the amount of acid, sugars and inorganic matter present in the powder while acid insoluble ash shows mainly silica thus indicating the contamination with earthy materials.[42] The extractive values are useful to evaluate the chemical constituents present in crude drug and also help for estimation of specific constituents soluble in a particular solvent. For any drugs and herbal formulation, low moisture content is essential for higher stability of drugs. The general requirement for moisture in a crude drug should not be more than 14%.[43] Excess moisture content may support the growth of fungi and may cause contamination by other microorganisms resulting into the degradation of drug. But HP powder showed 10% of moisture content i.e. loss on drying percentage (Table 2) which is not too high to encourage the growth of microorganisms. The pharmacological importance of a drug is attributed to the various secondary metabolites present in it and a particular compound might possess a clinical significance. Therefore it is essential to separate the compounds present in the plants with an appropriate chromatographic method. TLC technique has proved its worth as a simple, inexpensive and reproducible method for the chemical and biological screening of plant extracts. It provides a basic idea of polarity of a particular chemical constituent.[44] Development of TLC plates with appropriate group reagents indicates the presence of anthraglycosides, arbutin, flavonoids, cardiac glycosides and coumarins in HP powder. The pattern of bands on TLC plates provides fundamental data and is used to demonstrate the consistency and stability of herbal components. It is a potent and rapid way to distinguish between chemical classes which may not be fulfilled by macroscopic and microscopic analysis.[45] TLC is most recommended technique to create the fingerprints of herbal medicines because of its simplicity, versatility, specific sensitivity and easy sample preparation.[46] Thus, TLC is a convenient method of determining the quality and possible adulteration of herbal

Table 4: Preliminary phytochemical analysis of different solvent extracts of HP

	HPHx	HPHp	HPBz	HPEa	HPCl	HPAc	HPBu	HPEt	HPMt	HPAq
Tannin	-	-	-	-	-	-	-	-	-	-
Triterpenoids	-	-	-	-	-	+	+	+	+	+
Amino acid	-	-	-	-	-	-	-	-	-	+
Resin	-	-	-	-	-	-	-	-	-	-
Cardiac glycosides	+	+	+	++	+	++	+	+	++	++
Flavonoids	+	+	++	++	-	+++	+	+	+	+++
Alkaloids	-	-	-	-	-	-	-	-	-	-

(+++) appreciable amount; (++) moderate; (+) trace amount; (-) completely absent.

Table 5: *In vitro* antioxidant potential and quantitative phytochemical analysis of HP through successive solvents extraction

	DPPH IC_{50} (mg/ml)	ABTS IC_{50} (mg/ml)	SO IC_{50} (mg/ml)	NO IC_{50} (mg/ml)	MC IC_{50} (mg/ml)	FRAP mg AAE/mg EW	TPC (mg Gallic Acid eq./mg EW)	TFC (mg quercetine eq./mg EW)	TOPC (mg catechol eq./ mg EW)
HPHx	2.156 ± 0.148^{d}	0.171 ± 0.003^{f}	NA	2.990 ± 0.145^{a}	0.976 ± 0.084^{a}	9.311 ± 0.030^{g}	94.353 ± 0.074^{h}	12.627 ± 0.042^{b}	18.983 ± 0.011^{c}
HPHp	0.653 ± 0.003^{ab}	0.089 ± 0.001^{c}	NA	NA	0.328 ± 0.036^{a}	2.994 ± 0.006^{c}	361.413 ± 0.00^{a}	NA	88.859 ± 0.018^{a}
HPBz	1.094 ± 0.256^{bc}	$0.091 \pm 0.012c^{d}$	7.147 ± 0.073^{e}	NA	a	4.955 ± 0.088^{e}	139.2 ± 0.082^{d}	15.459 ± 0.045^{a}	14.128 ± 0.004^{e}
HPEa	0.465 ± 0.1667^{a}	0.062 ± 0.001^{b}	1.197 ± 0.276^{b}	3.516 ± 0.379^{a}	NA	1.889 ± 0.084^{b}	176.077 ± 0.238^{c}	7.326 ± 0.033^{d}	19.021 ± 0.005^{c}
HPCl	1.511 ± 0.057^{c}	0.065 ± 0.003^{b}	3.379 ± 0.079^{c}	NA	1.272 ± 0.061^{a}	9.931 ± 0.011^{h}	100.849 ± 0.046^{g}	NA	33.443 ± 0.02^{b}
HPAc	0.256 ± 0.034^{a}	0.037 ± 0.004^{a}	0.782 ± 0.311^{ab}	5.046 ± 0.476^{b}	4.278 ± 0.193^{c}	1.064 ± 0.029^{a}	272.988 ± 0.2^{b}	15.387 ± 0.071^{a}	17.406 ± 0.044^{d}
HPBu	0.612 ± 0.023^{a}	0.106 ± 0.012^{d}	0.833 ± 0.178^{ab}	9.224 ± 0.010^{d}	NA	3.544 ± 0.038^{d}	111.389 ± 0.088^{f}	NA	13.628 ± 0.019^{f}
HPEt	3.288 ± 0.594^{e}	0.249 ± 0.002^{g}	4.482 ± 0.511^{d}	6.832 ± 0.0568^{c}	NA	14.001 ± 0.12^{i}	24.419 ± 0.085^{j}	1.126 ± 0.006^{f}	5.902 ± 0.007^{h}
HPMt	1.243 ± 0.148^{c}	0.094 ± 0.006^{cd}	12.331 ± 0.158^{f}	NA	NA	6.942 ± 0.482^{f}	39.162 ± 0.263^{i}	1.716 ± 0.032^{e}	3.742 ± 0.022^{i}
HPAq	0.412 ± 0.015^{a}	0.134 ± 0.012^{e}	0.369 ± 0.151^{a}	29.608 ± 0.416^{c}	3.10 ± 0.946^{b}	1.726 ± 0.028^{b}	122.434 ± 0.168^{e}	10.053 ± 0.112^{c}	13.556 ± 0.077^{g}

Values with different letters (a, b, c, d, e, f, g, h & i) are significantly ($p < 0.05$) different from each other by Duncan's multiple range test (DMRT); NA: Not applicableAQ.

products. Over the ages, ethnomedicines have played a pivotal role in preventing and treating numerous human diseases and disorders. The credit of this ability may be given to the antioxidant potential of these plants used as ethnomedicine. For supporting the use of HP in traditional healing system, *in vitro* antioxidant activity of the various solvent extracts of HP was investigated. The previous studies have mentioned that no single method is sufficient to determine the antioxidant activity in a sample.[47] Hence various free radical scavenging activities of different solvent extracts of HP was evaluated. In DPPH, $ABTS^{+}$ and FRAP, acetone extract showed better activity along with high amount of TPC and TFC. Similar findings were noticed in *Bergenia ciliata* leaves where the acetone extract had exhibited significant free-radical scavenging property when compared with other non polar and polar compounds.[48] Heptane extract exhibited highest TPC and TOPC as compared to other extracts while TFC was found to be highest in the benzene extract. Chelation of metal, SO and NO scavenging activity was more in HPHp, HPHx and HPAq respectively. From the overall result, it was observed that the efficacy of each extract varied against different free radicals in specific assays indicating the complexity of mechanisms and the diverse chemical nature of the active phytoconstituents present in plants.[49] Qualitative phytochemical studies on the different solvent extracts of HP revealed that some solvents are good for extraction of phytoconstituents while some others are not so suitable. It is given in Table 4 that triterpenoids was present only in HPAc, HPBu, HPEt, HPMt and HPAq. Amino acids were found to be extracted only by the aqueous extract. Alkaloid was absent which was evidenced by the absence of Dragendorff's band in TLC method. All the extracts indicated the presence of cardiac glycosides and flavonoids particularly extracted by acetone and aqueous solvents in large amount. It could be assumed that the presence of the above phytoconstituents in some selected plant extracts alone or in combination might be credited for the antioxidant potential found in HP. To determine this view, Pearson correlation test was done between the phytochemical contents and the antioxidant activity of HP. The results in Table 6 showed a significant positive correlation between FRAP with DPPH and $ABTS^{+}$ while FRAP showed significant negative correlation with TPC indicating that phenolic content of HP might be responsible for causing ferric reducing potential. But there was no correlation between TPC, TFC and TOPC with the other radical scavenging activities of HP. Our observation supports the study of antioxidants on *Fraxinus floribunda* bark where no correlation was obtained between phenolics and antioxidant activity.[50] Similar finding was also reported in a number of medicinal plant extracts in which no correlation was found between total phenol content and antioxidant capacity.[51] Therefore it might be possible that there were other non-phenolic phytochemicals such as amino acids, uronic acids, ascorbic acid,

Table 6: Pearson's correlation coefficients between phytochemical content and free radical scavenging activity of HP

	DPPH	ABTS+	SO	NO	MC	FRAP	TPC	TFC
ABTS$^+$	0.826**	-	-	-	-	-	-	-
SO	0.367	0.099	-	-	-	-	-	-
NO	-0.302	0.084	-0.359	-	-	-	-	-
MC	-0.613	-0.347	-0.852	0.231	-	-	-	-
FRAP	0.964**	0.700*	0.422	-0.308	-0.586	-	-	-
TPC	-0.616	-0.554	-0.558	-0.129	0.044	-0.660*	-	-
TFC	-0.496	-0.482	-0.423	0.000	-0.044	-0.548	0.741	-
TOPC	-0.244	-0.263	-0.461	-0.259	-0.528	-0.220	0.781**	0.771*

**Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed).

tocopherol, pigments etc which could be responsible for the antioxidant capacity.[52,53] The strong positive correlation between DPPH and ABTS$^+$ suggests that the same phytoconstituents might be causing these free radicals scavenging activity of HP. TPC, TFC and TOPC were also found to be strongly correlated with each other.

CONCLUSION

All the scientific investigations support the traditional use of the powdered polyherb, HP for the treatment of heart problems which might be due to the accumulation of the free radicals. The Pearson correlation analysis suggested that the antioxidant potential possessed by HP was not due to phenolic compounds but it might be credited to other non-phenolic compounds found in plants. Further studies should be carried out in future using the animal models to confirm its antioxidant potential and its *in vivo* effectiveness of ethno-medicinal use. The data of pharmacognostic studies could be useful as a reference for the authentication and accurate identification of the polyherbal formulation, HP and also to differentiate it from its adulterants and alternatives.

ACKNOWLEDGEMENTS

We are thankful to the UGC for providing financial support under the scheme of Rajiv Gandhi National Fellowship. We are grateful to the Forests, Env. & W/L Management Dept. Govt. of Sikkim, Gangtok for providing the permit for survey in West Sikkim. Our heartfelt gratitude towards Mr. Nar Bahadur Subba, an herbal practitioner for providing us the sample and the traditional knowledge of the formulation.

CONFILCTS OF INTEREST

Authors declare no conflict of interest.

REFERENCES

1. Gangwar KK, Gangwar D, Gangwar RS. Ethnomedicinal plant diversity in Kumaun Himalaya of Uttarakhand, India. Nature and Science 2010; 8(5): 66-78.
2. Das T, Mishra SB, Saha D, Agarwal S. Ethnobotanical survey of medicinal plants used by ethnic and rural people in eastern Sikkim Himalayan region. African J Basic and Appl Sci. 2012; 4(1): 16-20.
3. Verma NS, Dwivedi S, Panigrahi D, Gupta SK. Anti-bacterial activity of root bark of *Nyctanthes arbor-tristis* Linn. Int J Drug Discovery and Herbal Res. 2011; 1(2): 61-2.
4. Dutta SC. Medicinal Plants. New Delhi: National Council for Education Research and Training; 1973.
5. Panda AK. Medicinal plants of Sikkim in Ayurvedic practice. Regional Research Institute (Ayurvedic) Gangtok, Sikkim. c2007- [Accessed: 2010 Apr 16]. Available from: http://www. sikenvis.nic.in/docs/Ayurvedhic%20 Medicines.pdf
6. Hussain W, Hussain J, Hussain S, Shinwari ZK, Ali R, Basir A. Ethono medicinal study of Parachinar, Kurram valley (fata) kpk, Pakistan. J App Pharm Sci. 2013; 3(11): 85-8.
7. Bhat JA, Kumar M, Rainer WB. Ecological status and traditional knowledge of medicinal plants in Kedarnath Wildlife Sanctuary of Garhwal Himalaya, India. J Ethnobiol Ethnomed. 2013; 9(1): 1-18.
8. Rajbhandari M, Mentel R, Jha PK, Chaudhary RP, Bhattarai S, Gewali MB, *et al.* Antiviral activity of some plants used in Nepalese traditional medicine. Evid Based Complement Alternat Med. 2009; 6(4): 517-22.
9. Ivanova D, Gerova D, Chervenkov T, Yankova T. Polyphenols and antioxidant capacity of Bulgarian medicinal plants. J Ethnopharmacol. 2005; 96(1): 145-50.
10. Kumar AD, Mazumder UK, Gupta M, Kumar GPS, Selvan VT. Evaluation of antioxidant and free radical scavenging activities of *Oxystelma esculentum* in various *in vitro* models. J Comp Integ Med. 2008; 5(1): 1-6.
11. Veerapur VP, Prabhakar KR, Parihar VP, Kandadi MR, Ramakrishana S, Mishra B, *et al. Ficus racemosa* stem bark extract: a potent antioxidant and a probable natural radioprotector. Evid Based Complement Alternat Med. 2009; 6(3): 317-24.
12. Ghosal M, Mandal P. *In vitro* antidiabetic and antioxidant activity of *Calamus erectus* Roxb. Fruit: a wild plant of Darjeeling himalaya. Int J Pharm Bio Sci. 2013; 4(2): 671-84.
13. Mandal P, Misra TK, Singh ID, Das JK, Bhunia M. Free-Radical–Scavenging activity in the inflorescence of European Nettle/Sisnu (*Urtica dioica* L.). J Young Pharm. 2009; 1(2): 129-35.
14. Mukhia S, Mandal Singh DK, Singh D, Choudhury D. *In vitro* free-radical scavenging potential of three liverworts of Darjeeling himalaya. Int J Pharm Sci Res. 2014; 5(10): 4552-61.
15. Antolovich M, Prenzler P, Robards K, Ryan D. Sample preparation in the determination of phenolic compounds in fruits. Analyst. 2000; 125(5): 989-1009.
16. Sultana B, Anwar F, Przybylski R. Antioxidant activity of phenolic components present in barks of *Azadirachta indica*, *Terminalia arjuna*, *Acacia nilotica*, and *Eugenia jambolana* Lam trees. Food Chem. 2007; 104(3): 1106-14.
17. Sultana B, Anwar F, Ashraf M. Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. Molecules 2009; 14(6): 2167-80.
18. Kokate CK, Purohit AP, Gokhale SB. Text book of Pharmacognosy. 18th ed. Pune: Nirali Prakashan; 2002.
19. Lala PK. Practical Pharmacognosy. 1st ed. Calcutta: Lina; 1981.
20. Chase CR, Pratt R. Fluorescence of powdered vegetable drugs with particular reference to development of a system of identification. J Am Pharmacol Assoc. 1949; 38 (6): 324-31.
21. Harborne JB. Phytochemical methods-A guide to modern techniques of plant analysis. Berlin: Springer Verlag; 2005.
22. Kumar A, Ilavarasan R, Jayachandan T, Decaraman M, Aravindhan P, Padmanabhan N. Phytochemical investigation on a tropical plant, *Syzygium cumini* from Kattuppalayam, Erode District, Tamil Nadu, South India. Pak J Nutr. 2009; 8(1): 83-5.
23. Ngbede J, Yakubu RA, Nyam DA. Phytochemical screening for active compound in *Canaruim schweinfurthii* (Atile) leaves from Ios North, Plateau state, Nigeria. Res J Biol Sci. 2008; 3(9): 1076-8.
24. Ibrahim HA, Ibrahim H. Phytochemical screening and toxicity evaluation on the leaves of *Argemone mexicana* Linn. (*Papaveraceae*). Int J Pure Appl Sci Technol. 2009; 3: 39-43.
25. Trease GE, Evans WC. Pharmacognosy. 13th ed. London: Bailliere Tuidal; 1989. p. 799-80.
26. Wagner H, Bladt S. Plant Drug Analysis: A Thin Layer Chromatography Atlas. 2nd ed. Berlin: Springer Verlag; 2009.
27. Chandler SF, Dodds JH. The effect of phosphate, nitrogen and sucrose on the production of phenolics and solasidine in callus cultures of *Solanum laciniatum*. Plant Cell Rep. 1983; 2(4): 205-8.

28. Kim DO, Jeong SW, Lee CY. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. Food Chem. 2003; 81(3): 321-6.
29. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 1958; 181: 1199-200.
30. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 1999; 26(9): 1231-7.
31. Nishikimi M, Appaji N, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun. 1972; 46(2): 849-54.
32. Marcocci L, Packer L, Droy-Lefaix MT, Sekaki A, Gardes-Albert M. Antioxidant action of *Ginkgo biloba* extract EGb 761. Methods Enzymol. 1994; 234: 462-75.
33. Dinis TC, Madeira VM, Almeida LM. Action of phenolic derivates (acetoaminophen, salycilate and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch Biochem Biophys. 1994; 315(1): 161-9.
34. Oyaizu M. Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucoamine. Jpn J Nutr. 1986; 4(6): 307-15.
35. World Health Organization. Quality control methods for medicinal plant materials WHO/PHARM/92.559; 1998. p. 4-46.
36. Anonymous. Indian Pharmacopoeia, Vol-II, Ministry of Health and Family Welfare, Govt of India. New Delhi: Controller of Publications; 1996.
37. Khandelwal KR. Practical pharmacognosy technique and experiments. 23rd ed. Pune: Nirali Prakashan; 2005. p. 149-56.
38. Purohit AP, Kokate CK, Gokhale SB. Pharmacognosy. 13th ed. Pune: Nirali Prakashan; 2005. p. 256-9.
39. Kumar HD, Mannem K. A comparison study of macroscopical and microscopical characteristics of powder of Haritaki: *Terminalia chebula* (pericarp), Yavani: *Ttrachyspermum ammi* (fruit), Ajmoda: *Apium leptophyllum* (fruit) and Sunthi: *Zingiber officinalie* (rhizome). Int J Ayurveda Pharma Res. 2012; 3(2): 309-13.
40. Pavanasasivam G, Suktanbawa MU. Flavonoids of some *Dilleniaceae* species. Phytochem. 1975; 14(4): 1127-8.
41. Janchen D, Issaq HJ. Modern thin layer chromatography: advances and perspectives. J Liquid Chromatogr. 1988; 11(9-10): 1941-65.
42. Ansari SH. Essentials of Pharmacognosy. 1st ed. New Delhi: Birla Publications Pvt. Ltd.; 2006.
43. African Pharmacopoeia, General methods for analysis. 1st ed. Vol 2. Lagos; 1986. p. 123.
44. Dutta J. Phytochemicals analysis and TLC fingerprinting of methanolic extracts of three medicinal plants. Int Res J Pharm. 2013; 4(6): 123-6.
45. Folashade KO, Omoregie EH, Ochogu AP. Standardization of herbal medicines: a review. Int J Biodivers Conserv. 2012; 4(3): 101-12.
46. Mohammad A, Bhawani SA, Sharma S. Analysis of herbal products by thin layer chromatography: A review. Int J Pharm Biosci. 2010; 1(2): 1-50.
47. Erel O. A novel automated method to measure total antioxidant response against potent free radical reactions. Clin Biochem. 2004; 37(2): 112-9.
48. Mandal P, Misra T. *In vitro* evaluation of free-radical scavenging potential of pakhenbed leaves [*Bergenia ciliata* (Haw.) Sternb.]. Int J Nat Eng Sci. 2009; 3(1): 01-5.
49. Vaijanathappa J, Badami S, Bhojraj S. *In vitro* antioxidant activity of *Enicostemma axillare*. J Health Sci. 2008; 54(5): 524-8.
50. Subba A, Mandal P. Antioxidant potential of *Fraxinus floribunda* bark extracted through various aqueous processing. Free Rad Antiox. 2015; 5(1): 6-12.
51. Sengul M, Yildiz H, Gungor N, Cetin B, Eser Z, Ercisli S. Total phenolic content, antioxidant and antimicrobial activities of some medicinal plants. Pak J Pharm Sci. 2009; 22(1): 102-6.
52. Chye FY, Wong JY, Lee JS. Nutritional quality and antioxidant activity of selected edible wild mushrooms. Food Sci. Technol. Int. 2008; 14(4): 375-84.
53. Perez-Jimenez J, Saura-Calixto F. Antioxidant capacity of dietary polyphenols determined by ABTS assay: a kinetic expression of the results. Int J Food Sci Tech. 2006; 43(1): 185-9.

ABOUT AUTHORS

Dr. Palash Mandal: Is an Assistant Professor at the Department of Botany, University of North Bengal, West Bengal, India. His research interest is in the area of Plant Physiology and Pharmacognosy. He has 48 nos. of research publications; 15 nos. of abstracts and 4 nos. of book chapters. He is having 13 years of teaching experience at North Bengal University and guiding many post graduate and doctoral students. Currently he has been working with bioactivities of plant peptides; antioxidant and anti-diabetic activities of various medicinal and aromatic plants.

Arunika Subba: Is a senior research fellow at the Department of Botany, University of North Bengal under the Rajiv Gandhi National Fellowship Scheme, UGC. She has one publication and her doctoral research is focused on the Pharmacognostic evaluation and *in vitro* antioxidant potential of some ethnomedicines used by Traditional Practitioners of West Sikkim.

Research Article

Antioxidant, anti-inflammatory, and hepatoprotective activity of *Fraxinus floribunda* bark and the influence of extraction process on their bioactivity

Arunika Subba[1], Bhaskar Dutta[2], Ram Kumar Sahu[3], Palash Mandal[1]*

ABSTRACT

Objective: To observe the influence of extraction processes on the bioactivity of *Fraxinus floribunda* (FF) bark on the basis of antioxidant activity and assess the *in vivo* anti-inflammatory with hepatoprotective activity in the extracts which has showed better antioxidant potential. **Materials and Methods:** Antioxidant activity on the basis of phytochemical estimation, free radical scavenging, and antilipid peroxidation activity. Anti-inflammatory activity was observed through carrageenan-induced paw edema, and hepatoprotective effect was studied using carbon tetrachloride induced liver damage in Wister albino rats. **Results:** Autoclave boiled extract showed highest antioxidant activity in assays of total phenol content (26.98 ± 11.22 mg gallic acid equivalent/g fresh weight tissue), 2,2-diphenyl-1-picryl hydrazyl (0.24 ± 3.65 mg/ml), etc. The same extract inhibited mice paw edema by 55% which was significant after 2 h ($P < 0.05$) showing anti-inflammatory activity. Lowering of serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, anti-lipid peroxidation, and bilirubin levels showed hepatoprotective activity against standard formulation Liv-52. **Conclusion:** The bark of FF is a potential source of antioxidants with anti-inflammatory and hepatoprotective activity. Further research should be done to explore and validate its bioactivity.

KEY WORDS: Anti-inflammatory, Antioxidants, *Fraxinus floribunda*, Hepatoprotection

INTRODUCTION

Plants have played a significant role in human health since ancient times. They are known to possess various biological effects including antioxidant, antitumor, antidiabetic, anti-inflammatory, hepatoprotective properties, etc. Recent trend in medicine looks forward for natural sources for the treatment of various ailments. Traditional medicine is supported by various researchers based on their therapeutic potential with less side effects and cost effectiveness. Plants are the source of natural antioxidants which can control oxidative stress resulting from the generation of free radicals. Oxidative stress if not controlled may cause various harmful diseases such as neurodenerative diseases (Alzheimer's and Parkinson's disease), arthritis, cancer, rheumatoid.[1,2] Metabolism of certain commonly used drugs such as paracetamol produce free radicals, which induces liver damage.[3,4] Since there are limited treatment and prevention for liver diseases, it is considered to be one of the most serious health problems in the world.[5] Inflammation is another least noticed health issue that is of big concern. It is a defensive response of a body against stress that is characterized by redness, pain, heat, and swelling along with loss of function in the injured area. However, if inflammation is left unchecked, it may lead to onset of several diseases such as rheumatoid arthritis, vasomotor rhinorrhea, and atherosclerosis. It has been observed that several anti-inflammatory, neuroprotective, digestive, antinecrotic, and hepatoprotective drugs have recently been shown to have an antioxidant or free radical scavenging potential as part of their activity.[6] Most of the modern medicines belong to steroidal or non-steroidal anti-inflammatory chemical

Access this article online	
Website: jprsolutions.info	ISSN: 0974-6943

[1]Department of Botany, Plant Physiology and Pharmacognosy Research Laboratory, University of North Bengal, Rajarammohanpur, Siliguri, West Bengal, India, [2]Department of Pharmacology, Dr. B C Roy College of Pharmacy and AHS, Meghnad Saha Sarani, Durgapur, West Bengal, India, [3]Department of Pharmacognosy, Columbia Institute of Pharmacy, Tekari, Raipur, Chattisgarh, India

***Corresponding author:** Palash Mandal, Department of Botany, Plant Physiology and Pharmacognosy Research Laboratory, University of North Bengal, Rajarammohanpur, Siliguri, West Bengal, India. Phone: +91-9434886123. E-mail: nbubotanypm@gmail.com

Received on: 12-06-2017; Revised on: 26-07-2017; Accepted on: 10-08-2017

therapeutics for the treatment of inflammation-related diseases. Although these drugs are very effective but long-term administration is required for treatment of severe diseases. Furthermore, these drugs have various adverse effects. However, naturally originated agents are shown to have very little side effects and are therefore preferred over chemical therapeutics. In recent days, the use of herbal natural products has gained attention among the world population. Many of the herbal supplements are claimed to assist in healthy lifestyle.[7]

Fraxinus floribunda (FF) Wallich belonging to *Oleaceae* family is a tree mainly found in the Eastern Himalayas, Khasi Hills, and Sikkim. The leaves and bark of the plant is considered as popular ethnomedicine used by the herbal healers in some villages of Sikkim for treating bone fracture, dislocation, and gout.[8] The leaves were found to possess potential anti-inflammatory and antinociceptive activity[9] but there was not a single report of anti-inflammatory activity on the bark of the plant in spite of being frequently used in folk medicine. Thus, this work has focused on the antioxidant activity of the bark of FF (BOFF) in its four differently processed extracts to compare the variation in their antioxidant activity through extraction methods. The extract showing better bioactivity was selected for further evaluation of *in vivo* anti-inflammatory and hepatoprotective activities.

MATERIALS AND METHODS

Plant Material

BOFF was collected from Sankhu, Dentam of West Sikkim. The herbarium of the plant was submitted in the NBU Herbarium which was identified by Dr. A.P. Das, Professor, Plant Taxonomy and Environmental Biology, Department of Botany, University of North Bengal. A voucher specimen, accession no. 9632/Tag no E.S.03 was preserved for future reference.

Chemicals and Reagents

Methanol, 2,2-diphenyl-1-picryl hydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium ferricyanide (K3Fe(CN)6), nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide sodium salt monohydrate (NADH), sulfanilamide, glacial acetic acid, naphthyl ethylenediamine dihydrochloride, trichloroacetic acid (TCA), sodium hydroxide (NaOH), ferrous chloride ($FeCl_2$), thiobarbituric acid (TBA), trichloroacetic acid, $FeSO_4.7H_2O$, potassium hydroxide, potassium dihydrogen orthophosphate (KH_2PO_4), ethylene-diamine tetra acetic acid, 2-deoxyribose, Folin–Ciocalteu reagent, ferric chloride ($FeCl_3$), ferrozine, sodium nitroprusside, gallic acid, catechol, quercetin, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), aluminium chloride ($AlCl_3$), hydrochloric acid, and potassium chloride were used for antioxidant activity. Carbon tetrachloride (CCl_4), L-alanine, 2,4 dinitro phenyl hydrazine, sodium pyruvate, L-aspartic acid, and aspirin. All these chemicals were either purchased from Himedia, Sigma and Merck, Germany made.

Plant Sample Preparation

The fresh BOFF was sun dried for 4-5 days. The dried bark was ground into fine powder. The powder specimen was then extracted through four different methods, namely, normal boiling (NB), boiling in autoclave under pressure (AB), soxhlation in a soxhlet apparatus (S), and cold percolation below −4°C (CP). This method was repeated for three successive times to observe the retention of the bioactivity of compounds in the extracts.

Animals

Wister albino rats (150-250 g) were used in this study. They were obtained from Animal House of Columbia Institute of Pharmacy, Raipur. They were housed in large propylene cage and kept 22 ± 2°C in 12 h dark light cycle of 12:12 h, relative humidity 55-65%. The animals were fed with pellet food and water. The experiments were approved by Institutional Animal Ethics Committee of Columbia Institute of Pharmacy, Raipur, India (Regd. No. 1321/PO/ReBi/S/10/CPCSEA). All the animals were acclimatized for at least 1 week before the experimental session.

Antioxidant Activity

DPPH scavenging assay

Antioxidant activity was evaluated using DPPH.[10] The absorbance of color scavenging by the sample was measured at 517 nm in ultraviolet-visible spectrophotometer. Inhibitory concentration 50% (IC_{50}) was calculated with the percentage inhibition versus concentration graph. IC_{50} value is the effective concentration that could scavenge 50% of the DPPH radicals. Inhibition percentage was calculated with the following formula.

Percentage inhibition = [Absorbance of DPPH solution by control – Absorbance of DPPH solution by plant extract/Absorbance of control] ×100

$ABTS^+$ scavenging assay

$ABTS^+$ free radical scavenging activity of the sample was determined according to the method of Re *et al*.[11] Absorbance was taken at 734 nm. Percentage inhibition and IC_{50} values were calculated.

Nitric oxide scavenging assay (NO)

Aqueous solution of sodium nitroprusside generates NO at physiological pH. The NO again interacts with

oxygen to produce nitrite which can be observed by Greiss reaction at 540 nm.[12] Percentage inhibition was calculated to measure the NO radical scavenging activity of the sample.

Superoxide scavenging activity (SO)

SO scavenging activity of the aqueous sample was done by the method of Chou *et al*.[13] The mixture of NBT chloride, NADH, and PMS along with the sample extract was exposed to fluorescence light for 30 min. Absorbance of color scavenged by the samples was measured at 560 nm and percentage inhibition was calculated.

Reducing power (RP)

The aqueous extract (1 ml) was mixed with 2.5 ml buffer (0.3 M), 2.5 ml potassium ferricyanide, and left for incubation at 50°C for 20 min. To the reaction mixture, 2.5 ml TCA was added which was left to cool down and then it was centrifuged. The upper layer (2.5 ml) was collected and 2.5 ml of water was mixed with it followed by addition of the 250 µl ferric chloride. The absorbance was taken at 700 nm.[14]

Metal chelating activity

This method was performed to estimate the chelating activity of the aqueous sample extract for ferrous ions Fe^{2+}.[15] Aqueous extract of 400 µl was diluted in 1600 µl methanol followed by the addition of 40 µl ferrous chloride (2 mM). Ferrozine (80 µl) was added to the mixture and left it for incubation for 10 min. Optical density (OD) value was taken at 562 nm. Percentage inhibition was calculated.

Anti-lipid peroxidation assay (ALP)

The thiobarbituric acid reactive species assay was used to measure $FeSO_4$-induced lipid peroxidation in liver homogenates.[16] The oxidation of polyunsaturated fatty acids when reacts with two molecules of thiobarbituric acid (TBA) forms malondialdehyde yielding a pinkish-red which is measured with an absorbance at 532 nm.

Quantitative Estimation of Phytochemicals

Total phenol content (TPC)

Quantitative estimation of TPC was done by the method using Folin–Ciocalteu reagent.[17] Absorbance was taken at 725 nm and the phenol content was calculated as gallic acid equivalent/g fresh weight tissue (FWT).

Total flavonoids content (TFC)

TFC was determined as per the method of Sultana *et al*.[18] The aqueous extract was diluted in distilled water followed by the addition of sodium nitrate, aluminium chloride, and sodium hydroxide. The reaction mixture was vortexed and OD value was taken at 510 nm. TFC was calculated as quercetin equivalent/g FWT.

Total orthodihydric content (TOPC)

TOPC was estimated using Arnow's reagent.[19] Absorption of pink coloration was measured at 515 nm. The total content was measured as catechol equivalent/g FWT.

In vivo Pharmacological Activity

Acute toxicity study

The acute toxicity study of aqueous extract of BOFF was determined as per the CPCSEA guideline no 420 (fixed dose method). The aqueous bark extracts were orally given to the rats at the dose level of 5, 50, 300, and 1000 mg/kg, respectively. It was observed that the test showed no mortality even at maximum dose, i.e., 1000 mg/kg body weight (b.w.). Hence, $1/10^{th}$ (100 mg/kg) of this dose was selected for further study.[20]

Anti-inflammatory activity of carrageenan-induced paw edema (acute model)

Anti-inflammatory activity was performed by the method described by Winter and Porter.[21] Acute inflammation was produced by injecting 1% solution of carrageenan into planter surface of rat hind paw at the dose of 0.1 ml/100 g b.w. Wistar albino rats were divided into three groups of six in each. A 0.9% solution of NaCl at a dose of 0.1 ml/100 g p.o. was administered to Group 1 (control). The test drug sample BOFF was administered to animals of Group 2 (FF) at 100 mg/kg b.w. p.o. against the standard drug aspirin at the dose of 100 mg/kg b.w. p.o. to the Group 3 (aspirin). After 30 min, carrageenan solution was injected to the animals of the entire group. The paw edema was measured at the intervals of 30, 60, 120, and 180 min using plethysmometer. The paw edema among different groups was compared, the percentage inhibition of paw edema was determined by the formula, percentage inhibition of paw edema= $(V_c - V_t)/V_c) \times 100$

V_c = Paw edema of control animals,
V_t = Paw edema of drug-treated animals.

Hepatoprotective activity

The hepatoprotective activity in the aqueous extract of FF bark was determined by CCl_4-induced hepatotoxicity. Acute toxicity studies were conducted using Wistar rat. The animals were fasted overnight before the experiment procedure. The purified water was used as a vehicle to dissolve the extracts. Wistar rats (150-200 g) of either sex were used for hepatoprotective activity. The rats were divided into four groups with six animals in each which were maintained on standard diet and water *ad libitum*.

Group 1 (CONT) was given purified water only; Group 2 normal control (NOR CONT) were kept at the dose of 1 ml/kg as a vehicle for 10 days only by oral route. Aqueous extract of BOFF were administered to Group 3 (FF) at a dose of 100 mg/kg by oral route for 10 days, Group 4 (Liv-52) was fed with marketed formulation, Liv-52 syrup only at the dose of 2.5 ml/kg b.w. by oral route for 10 days only.[22] Liv-52 is a popular poly herbal formulation which contains Ayurvedic herbal remedy against viral hepatitis. It is widely prescribed for infective hepatitis.[23] CCl_4 at a dose of 0.75 ml/kg b.w. was injected to Group 1, 3, and Group 4 on 3rd, 6th, and 10th day by intraperitoneum route. On 10th day, 1 h after the last dose of CCl_4 injection, the blood was collected by retro-orbital route. The blood sample was centrifuged and used for the estimation of various biochemical parameters (serum glutamic oxaloacetic transaminase [SGOT], serum glutamic pyruvic transaminase [SGPT], ALP, and bilirubin levels) which are the markers of liver damage.

Statistical Analysis

The standard software SPSS was used for different statistical analysis. The values were expressed as mean ± standard error of mean. The data were analyzed using Tukey's test. The means with $P < 0.05$ were considered as significant.

RESULTS

The aqueous extracts of BOFF showed potential antioxidant activity but we have focused more on the variation of bioactivity due to the change in extraction processes of the sample.

The box plots (Figures 1-10) clearly illustrate the changes in the bioactivity as well as retention of this activity in various processes after their successive extraction. From the box plots, we have observed that soxhlation process could extract a concentrated amount of bioactive components at the 1st stage of boiling which then rapidly decreases in the 2nd and the 3rd stages due to which the IC_{50} values in DPPH scavenging activity ranges from low (2.17 mg/ml) in the 1st stage to high (486.61 mg/ml) in the 3rd stage. Similar results were found in $ABTS^+$ (Figure 2), SO (Figure 3), NO scavenging activity (Figure 4) along with metal chelating activity (Figure 5) and RP (Figure 7). In case of normal boiling, autoclave boiling and cold percolation, the bioactivity has gradually decreased from 1st to 3rd stage showing more retention and gradual extraction of bioactive compounds from the plant samples. Among all the processes, autoclave boiling has showed to be an ideal process for extraction of bioactive compounds from the BOFF as it has showed more antioxidant activity on the basis of DPPH, $ABTS^+$, NO, ALP, RP,

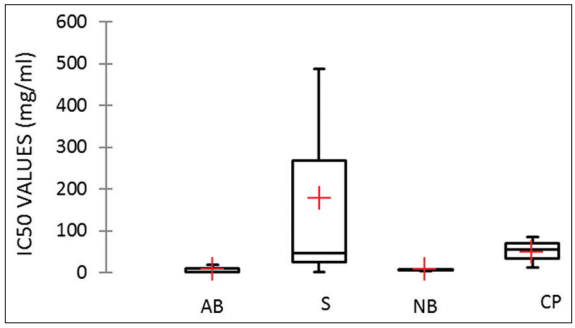


Figure 1: 2,2-diphenyl-1-picryl hydrazyl radical scavenging activity. AB: Autoclave boiling, S: Soxhlation, NB: Normal boiling, CP: Cold percolation

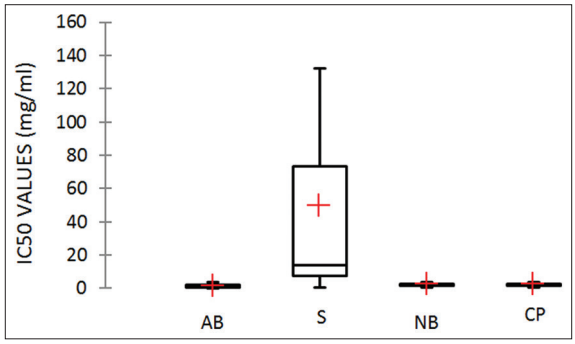


Figure 2: 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical scavenging activity. AB: Autoclave boiling, S: Soxhlation, NB: Normal boiling, CP: Cold percolation

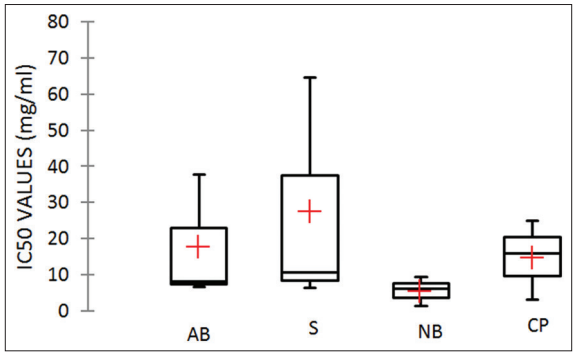


Figure 3: Superoxide scavenging activity. AB: Autoclave boiling, S: Soxhlation, NB: Normal boiling, CP: Cold percolation

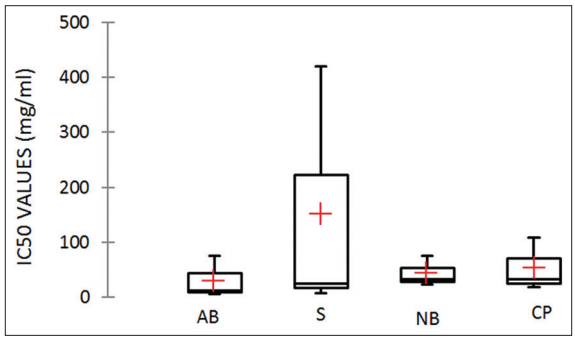


Figure 4: Nitric oxide scavenging activity. AB: Autoclave boiling, S: Soxhlation, NB: Normal boiling, CP: Cold percolation

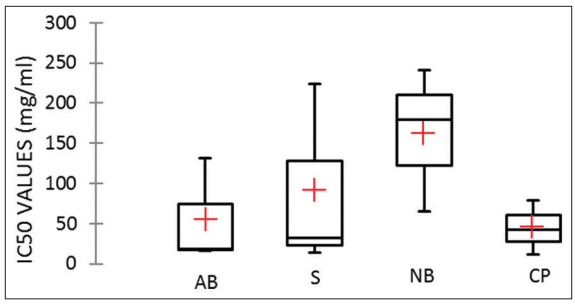


Figure 5: Metal chelating activity. AB: Autoclave boiling, S: Soxhlation, NB: Normal boiling, CP: Cold percolation

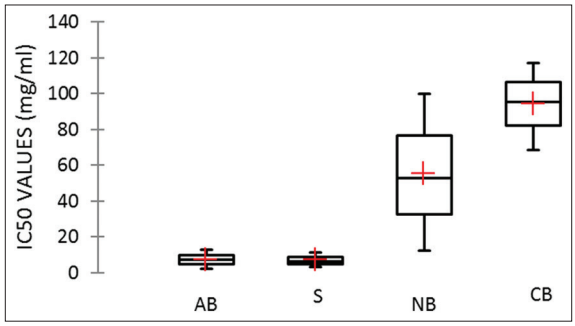


Figure 6: Anti-lipid peroxidation activity. AB: Autoclave boiling, S: Soxhlation, NB: Normal boiling, CP: Cold percolation

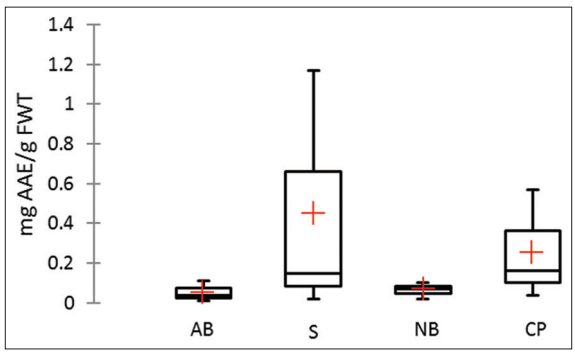


Figure 7: Reducing power activity. AB: Autoclave boiling, S: Soxhlation, NB: Normal boiling, CP: Cold percolation

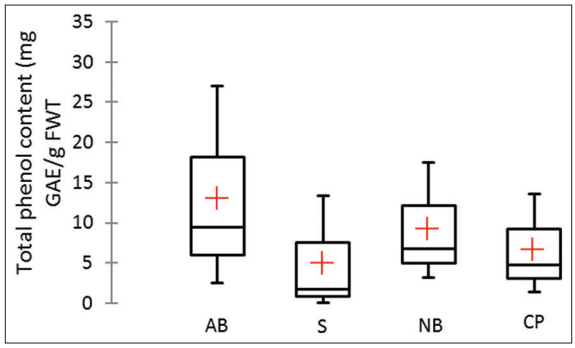


Figure 8: Total phenol content. AB: Autoclave boiling, S: Soxhlation, NB: Normal boiling, CP: Cold percolation

TPC, and TFC. It has also showed higher retention of bioactivity until 3rd stage of extraction. Therefore, extract obtained from autoclave boiling was further

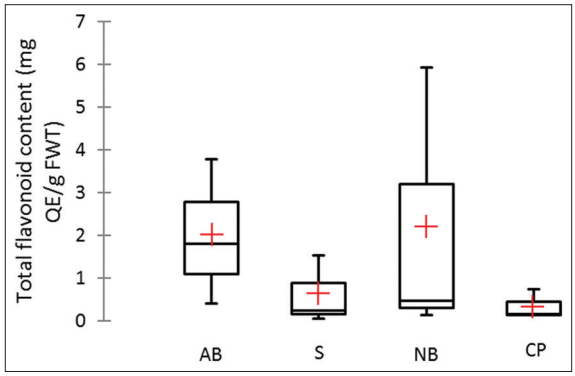


Figure 9: Total flavonoid content. AB: Autoclave boiling, S: Soxhlation, NB: Normal boiling, CP: Cold percolation

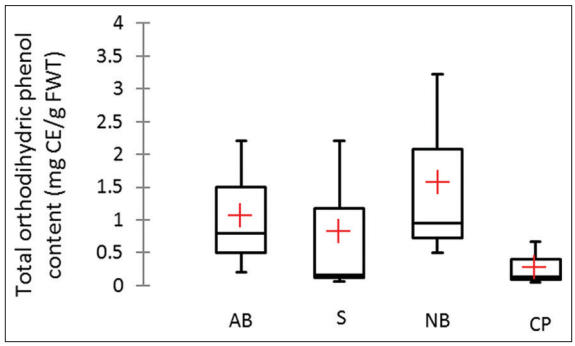


Figure 10: Total orthodihydric phenol content. AB: Autoclave boiling, S: Soxhlation, NB: Normal boiling, CP: Cold percolation

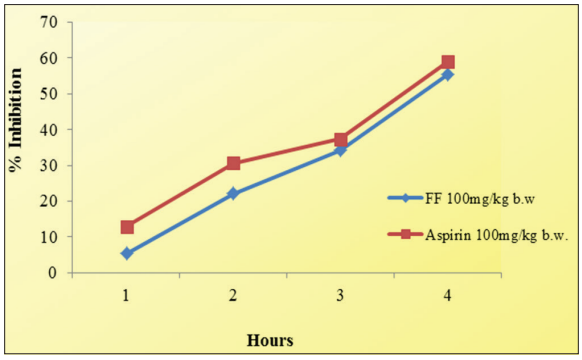


Figure 11: Percentage inhibition of paw oedema by bark of *Fraxinus floribunda* extract compared with aspirin

subjected for *in vivo* assays such as anti-inflammatory and hepatoprotective activity. The process of carrageenan-induced mice paw edema has two phases. During early hyperemia, which occurs 0-2 h after the carrageenan injection, there is an increase in vascular permeability due to the release of histamine, serotonin, and bradykinin. The inflammatory edema reaches its highest level after 2 h and then will start to decline. In our study, paw edema was increased and reached a maximum at 2 h after carrageenan injection. Treatment with FF bark extract at the 100 mg/kg b.w. significantly ($P < 0.05$) reduced paw edema formation (0.833 ± 0.01 ml) after 2 h as shown in Table 1. Percentage inhibition of paw edema was 55% by the

Table 1: Effect of aqueous extract of BOFF on anti-inflammatory activity on carrageenan rat edema with time and percentage inhibition

Dose	30 min	60 min	120 min	180 min
NaCl control Gr	1.59±0.07^{b}	1.84±0.05^{c}	1.9±0.04^{b}	1.86±0.06^{b}
Standard Gr (aspirin) 100 mg/kg b.w.	1.37±0.02^{a}	1.28±0.06^{a}	1.19±0.05^{a}	0.77±0.04^{a}
% Inhibition	12.9	30.6	37.3	59
Fraxinus floribunda 100 mg/kg b.w.	1.5±0.02^{b}	1.43±0.02^{b}	1.25±0.04^{a}	0.833±0.01^{a}
% Inhibition	5.2	22.1	34.1	55

Values are expressed as mean±SEM (n=6). P values were analyzed using Tukey's test. P<0.05 were considered to be significant when treated groups were compared with the control group, a more significant than b and c. SEM: Standard error of mean, BOFF: Bark of *Fraxinus floribunda*, b.w.: Body weight

BOFF which was close to the standard drug aspirin (100 mg/ml) as presented in Figure 11. To evaluate the hepatoprotective activity of BOFF, liver damage and recovery from damage was assessed on 10th day by measuring serum marker enzymes to evaluate the biochemical changes in liver. It is well-known that hepatotoxicity by CCl_4 is due to enzymatic activation to release free radical state. CCl_4 is biotransformed in liver by cytochromes P450 enzyme to form CCl_3 active free radical with oxygen, which then covalently binds with cellular macromolecules and creates loss of cellular integrity and hepatic damage. CCl_4 causes a range of histological changes to the liver including inflammation and cellular swelling. Therefore, antioxidant and anti-inflammatory efficacy are regarded as some important parameters indicating possible mechanism of hepatoprotection. The increased level of SGOT, ALP, SGPT, and bilirubin is conventional indicator of liver injury.[24-26] In this study, it was found that administration of CCl_4 elevated the levels of serum marker enzymes SGOT, ALP, SGPT, and serum bilirubin in CONT Gr as represented in Figures 12-15, respectively. The experimental results of pretreatment with BOFF through autoclave boiling and Liv-52 treated groups showed to lower the levels of SGPT, SGOT, ALP, and bilirubin as compared to CCl_4-treated group indication the inhibition of CCL_4-induced elevation of SGOT, SGPT, ALP, and bilirubin levels in hepatic rats.

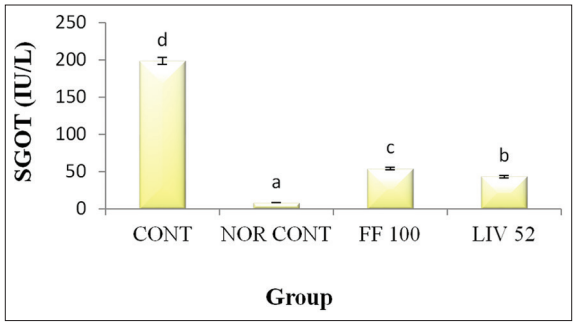


Figure 12: Effect of bark of *Fraxinus floribunda* extracts on serum glutamic oxaloacetic transaminase level in hepatotoxic rats. Values are expressed as mean ± standard error mean. P values were analyzed using Tukey's test. $P < 0.05$ was considered to be significant when treated groups were compared with the control group. FF: Aqueous extract of *Fraxinus floribunda* bark

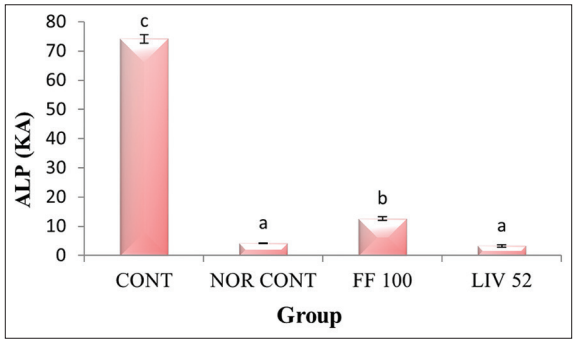


Figure 13: Effect of bark of *Fraxinus floribunda* extracts on anti-lipid peroxidation level in hepatotoxic rats. Values are expressed as mean ± standard error mean. P values were analyzed using Tukey's test. $P < 0.05$ was considered to be significant when treated groups were compared with the control group. FF: Aqueous extract of *Fraxinus floribunda* bark

DISCUSSION

Plants are storehouse of various secondary metabolites which could be used as a natural source for developing novel drugs in the future. These secondary metabolites could be extracted from plants through different process variation and the type of solvents as well as extraction process could influence the bioactivity of these plants.[27,28] The plant sample we have collected, i.e., the BOFF was a commonly used ethnomedicine which was boiled in water until the color and the bitterness in the water was gone completely. In our study, we have tried to observe the influence of variation in the extraction process on the antioxidant activity of the sample as well retention of this bioactivity after successive extraction. The retention of bioactivity by AB and NB extracts as given in the box plots might be due to less time duration taken for extraction while soxhlation process has showed sudden decrease in bioactivity because this method took 6-8 h for extraction. The long duration of time might have been able to extract most of the bioactive compounds from the plant sample at the initial stage itself. Thus, pressure boiling method could be recommended for the extraction of antioxidants from the BOFF because it has showed better activity, less time-consuming, and retention of the activity for a longer duration.

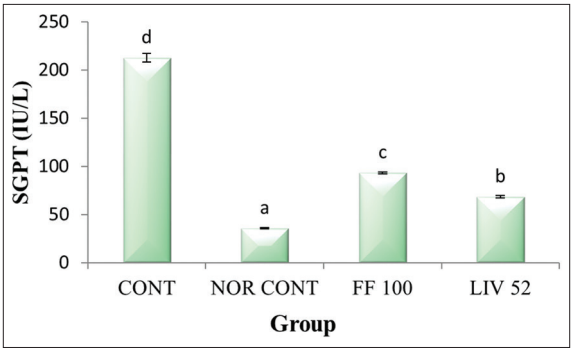


Figure 14: Effect of bark of *Fraxinus floribunda* extracts on serum glutamic pyruvic transaminase level in hepatotoxic rats. Values are expressed as mean ± standard error mean. *P* values were analyzed using Tukey's test. $P < 0.05$ was considered to be significant when treated groups were compared with the control group. FF: Aqueous extract of *Fraxinus floribunda* bark

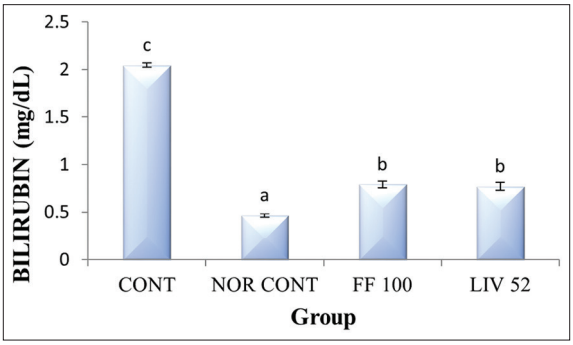


Figure 15: Effect of bark of *Fraxinus floribunda* extracts on bilirubin level in hepatotoxic rats. Values are expressed as mean ± standard error mean. *P* values were analyzed using Tukey's test. $P < 0.05$ was considered to be significant when treated groups were compared with the control group. FF: Aqueous extract of *Fraxinus floribunda* bark

Inflammation is condition which is caused due to the reaction of living tissues during injury.

In this study, we have used the carrageenan-induced rat paw edema model for anti-inflammatory study which is believed to be a biphasic process. The initial phase of 1-2 h inflammation is induced by carrageenan due to the synthesis of serotonin, histamine, and prostaglandins in the surrounding of the injured tissue. The second phase is continued by prostaglandin synthesis and mediated by leukotrienes, bradykinin, prostaglandins, and polymorphonuclear cells produced by tissue macrophages.[29] The results showed that standard drug aspirin as well as BOFF extract (100mg/kg b.w.) showed significant anti-inflammatory activity at later stages after 2 h (Table 1). Since flavonoids are known to prevent prostaglandins synthesis, the anti-inflammatory activity of BOFF might be credited to the flavonoids present in the sample as indicated in quantitative estimation of total flavonoids.[30,31] Prophylactic use of the extract of FF resulted in an inhibition of degree of hepatic necrosis and along with decrease of the intercellular enzyme leakage by stabilizing hepatic cellular membrane which may be due to its highest antioxidant and anti-inflammatory property of the same plant as confirmed by our study. The evaluation of pharmacological assays showed that autoclave boiling extract of BOFF was good hepatoprotective agent at a dose level of 100 mg/kg b.w. when compared with standard formulation Liv-52.

This study revealed that autoclave boiled extract of FF bark exhibited highest antioxidant activity as well as retained its activity for a longer time. This extracts when was further subjected to *in vivo* anti-inflammatory and hepatoprotective activity showed effective results which were compared with the respective standards. The anti-inflammatory and hepatoprotective effects seem to be related to a modulation of antioxidant enzyme activity in the liver. The hepatoprotective mechanisms of FF when orally administered seemed to involve in maintaining the liver antioxidative defense systems in addition with scavenging of free radicals. Thus the BOFF acts as a pharmacological agent which can prevent inflammatory and liver disorders.

ACKNOWLEDGMENT

We are grateful to the University Grants Commission for providing financial support through Rajiv Gandhi National Fellowship. We would also like to acknowledge the Forests, Environment and W/L Management Department, Government of Sikkim, Gangtok for providing the permit for survey in West Sikkim. Our heartfelt gratitude toward Mr. Tulshi Ram Sharma, an herbal practitioner for providing us the plant sample and its traditional value.

REFERENCES

1. Scalbert A, Manach C, Morand C, Rémésy C, Jiménez L. Dietary polyphenols and the prevention of diseases. Crit Rev Food Sci Nutr. 2005;45(4):287-306.
2. Olukemi OA, Olukemi IO, Sofidiya MO, Aniunoh OA, Lawal BM, Tade IO. Antioxidants activity of Nigerian dietary spices. Electron J Environ Agric Food Chem. 2005;496:1086-93.
3. Jaeschke H. Reactive oxygen and mechanisms of inflammatory liver injury: Present concepts. J Gastroenterol Hepatol. 2011;26 Suppl 1:173-9.
4. Kaplowitz N. Biochemical and cellular mechanisms of toxic liver injury. Semin Liver Dis. 2002;22(2):137-44.
5. Amat N, Upur H, Blazekovic B. *In vivo* hepatoprotective activity of the aqueous extract of *Artemisia absinthium* L. against chemically and immunologically induced liver injuries in mice. J Ethnopharmacol. 2010;131(2):478-84.
6. Rop O, Mlcek J, Jurikova T, Neugebauerova J, Vabkova J. Edible flowers-a new promising source of mineral elements in human nutrition. Molecules. 2012;17(6):6672-83.
7. Li R, Guo W, Fu Z, Ding G, Zou Y, Wang Z. Hepatoprotective action of *Radix paeoniae rubra* aqueous extract against CCl4-induced hepatic damage. Molecules. 2011;16(10):8684-94.
8. Bijoy G. The Medicinal Plants of the Sikkim Himalaya. Chakung: Maples; 2002.

9. Lingadurai S, Nath LK, Kar PK, Besra SE, Joseph RV. Anti-inflammatory and anti-nociceptive activities of methanolic extract of the leaves of *Fraxinus floribunda* Wallich. Afr J Tradit Complement Altern Med. 2007;4(6):411-6.
10. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958;181:1199-200.
11. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 1999;26(9-10):1231-7.
12. Patel DK, Kumar R, Laloo D, Hemalatha S. Evaluation of phytochemical and antioxidant activities of the different fractions of *Hybanthus enneaspermus* (Linn.) F. Muell. (*Violaceae*). Asian Pac J Trop Med. 2011;4(5):391-6.
13. Chou HG, Kuo JT, Lin ES. Comparative antioxidant properties of water extracts from different parts of beefsteak plant (*Perilla frutescens*). J Food Drug Anal. 2009;17:489-6.
14. Oyaizu M. Studies on products of browning reactions: Antioxidative activities of products of browning reaction prepared from glucoamine. Jpn J Nutr. 1986;4:307-15.
15. Dinis TC, Madeira VM, Almeida LM. Action of phenolic derivates (acetoaminophen, salycilate, and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch Biochem Biophys. 1994;315(1):161-9.
16. Bouchet N, Barrier L, Famconneau B. Radical scavenging activity and antioxidant properties of tannins from *Guiera senegalensis* (*Combretaceae*). Phytother Res. 1998;12:159-62.
17. Stankovic MS. Total phenolic content, flavonoid concentration and antioxidant activity of *Marrubium peregrinum* L. extracts. Kragujevac J Sci. 2011;33:63-72.
18. Sultana B, Anwar F, Ashraf M. Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. Molecules. 2009;14:2167-80.
19. Kim DO, Jeong SW, Lee CY. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. Food Chem. 2003;81:321-6.
20. Hanafy A, Aldawsari HM, Badr JM, Ibrahim AK, Abdel-Hady SE. Evaluation of hepatoprotective activity of *Adansonia digitata* extract on acetaminophen-induced hepatotoxicity in rats. Evid Based Complement Altern Med. 2016;2016:4579149.
21. Winter CA, Porter CC. Effect of alterations in side chain upon anti-inflammatory and liver glycogen activities of hydrocortisone esters. J Am Pharm Assoc Am Pharm Assoc. 1957;46(9):515-9.
22. Tasaduq SA, Singh K, Sethi S, Sharma SC, Bedi KL, Singh J, *et al.* Hepatocurative and antioxidant profile of HP-1, a polyherbal phytomedicine. Hum Exp Toxicol. 2003;22(12):639-45.
23. Mukerjee AB, Dasgupta M. Treatment of viral hepatitis B an indegenious drug Liv-52. Indian Pract. 1970;6:357.
24. Grigonisa D, Venskutonis PR, Sivik B, Sandahl M, Eskilsson CS. Comparison of different extraction techniques for isolation of antioxidants from sweet grass (*Hierochloe odorata*). J Supercrit Fluids. 2005;3:223-33.
25. Michiels JA, Kevers C, Pincemail J, Defraigne JO, Dommes J. Extraction conditions can greatly influence antioxidant capacity assays in plant food matrices. Food Chem. 2012;130:986-93.
26. Lee CP, Shih PH, Hsu CL, Yen GC. Hepatoprotection of tea seed oil (*Camellia oleifera* Abel.) against CCl4-induced oxidative damage in rats. Food Chem Toxicol. 2007;45(6):888-95.
27. Recknagel RO, Glende EA Jr, Dolak JA, Waller RL. Mechanisms of carbon tetrachloride toxicity. Pharmacol Ther. 1989;43(1):139-54.
28. Weber LW, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: Carbon tetrachloride as a toxicological model. Crit Rev Toxicol. 2003;33(2):105-36.
29. Brito AR, Antonio MA. Oral anti-inflammatory and antiulcerogenic activities of a hydroalcoholic extract and partitioned fractions of *Turnera ulmifolia* (*Turneraceae*). J Ethnopharmacol. 1998;61:215-28.
30. Middleton E Jr, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. Pharmacol Rev. 2000;52(4):673-751.
31. Havsteen BH. The biochemistry and medical significance of the flavonoids. Pharmacol Ther. 2002;96(2-3):67-202.

ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 12, Issue 5, 2019

Online - 2455-3891
Print - 0974-2441

Research Article

STANDARDIZATION OF A TRADITIONAL POLYHERBAL FORMULATION WITH PHARMACOGNOSTIC STUDY; ITS PHYTOCHEMICAL CONTENT, ANTIOXIDANT, AND ANTIDIABETIC ACTIVITY

ARUNIKA SUBBA, PALASH MANDAL*

Plant Physiology and Pharmacognosy Research Laboratory, Department of Botany, University of North Bengal, Rajarammohunpur, Shivmandir, Siliguri, West Bengal, India. Email: pmandalbotppprl@nbu.ac.in

Received: 21 February 2019, Revised and Accepted: 03 April 2019

ABSTRACT

Objective: Pharmacognostic study, evaluation of antioxidant and antidiabetic activity along with phytochemical contents of an ethnomedicine AR as it is mainly used for the treatment of arthritis and diabetes by some healers in the villages of West Sikkim.

Methods: The herbal formulation was extracted in a Soxhlet apparatus successively in ten solvents from low to high polarity. The extracts were subjected to antioxidant activity, qualitative and quantitative phytochemical estimation as well as *in vitro* antidiabetic activity. For pharmacognostic characterization, parameters such as fluorescence activity, physicochemical values, powder microscopy, and thin-layer chromatography (TLC) were performed. Mean values with $p<0.05$ were considered significant in statistical analysis.

Results: Pharmacognostic study revealed various plants tissues. Ash values suggested the presence of earthy materials and moisture content near to the maximum range. Variation of colors was exhibited by AR in fluorescence analysis. TLC expressed the presence of phytoconstituents and the R_f values were noted down to be used in the future for authentication of the sample. Potential antioxidant capacity was observed in AR, phenolics significantly contributing in 1,1-diphenyl-2-picrylhydrazyl scavenging activity, 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)$^+$ scavenging activity and reducing power. Non-polar solvent showed the presence of alkaloid and steroids. The antidiabetic activity was very high in some extracts of AR with acetone extract showing the highest enzyme inhibiting activity. IC_{50} of acetone extract was 0.26±0.003 mg/ml.

Conclusion: Overall study established a basic reference for the formulation AR. It was considered to possess antioxidant activity, but the interesting part of the study was its antidiabetic activity which is needed to be validated with *in vivo* studies and toxicity assessment.

Keywords: Ethnomedicine, Pharmacognosy, Antioxidant, Antidiabetic, Phytochemical.

 DOI: http://dx.doi.org/10.22159/ajpcr.2019.v12i5.32725

INTRODUCTION

Medicinal plants have always been an important part of human life. Numerous plants have established its identity in the development of novel drugs. The synthetic drugs are effective and have dominated the world of medicine, but it has also brought harmful side effects along with it. Natural sources of drugs are the only alternative to this problem which can be fulfilled through the search of medicinal plants with potential therapeutic activity. Reactive oxygen species (ROS) such as superoxide (SO) ions and hydroxyl radicals, and hydrogen peroxide when accumulated in the body initiates the development of various degenerative or pathological processes including aging, cancer, heart disease, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, and diabetes [1]. Antioxidants control the damage caused by ROS which scavenges and reduces the formation of free radicals [2]. The therapeutic property of several medicinal plants is generally credited to the secondary metabolites present in them bearing antioxidant activity. In fact, dietary intake of antioxidant-rich food is believed to be inversely related to the human disease [3]. Butylated hydroxytoluene and butylated hydroxyanisole are common synthetic antioxidants which along with beneficial properties give side effects that might cause health hazards in a long-term basis. Plant-based antioxidants are preferred over synthetic ones due to safety concern [4]. Hence, research regarding plants rich in antioxidants is vital as it not only provides information about antioxidant supplement but also helps in the selection of plants for the development of novel drugs from natural sources.

Sikkim is a small Himalayan state of Northeast India which is also considered to possess numerous medicinal plants with a large number of indigenous people still depending on traditional medicine mainly from plant sources. In the present study, we have collected a polyherbal formulation from an herbal practitioner of the West district of Sikkim which was used for the treatment of arthritis and high blood sugar. We have abbreviated the formulation as "AR." It was prepared from three plants including the whole plant of *Viscum articulatum* Burm. f., rhizome of *Rheum acuminatum* Hooker. f., and Thomson and rhizome of *Astilbe rivularis* Buch.-Ham.ex D. Don. These plant parts were mixed and stored in powder form. It is a common fact that the collection of medicinal plants often faces the challenge of standardization. Since the therapeutic value of medicinal plants depends on the quality and quantity of chemical constituents, it is essential to standardize each and every ethnomedicine used by man. This can be achieved by a pharmacognostic study which includes parameters that help to authenticate the drug even in dry powder form after losing its morphological characteristics where there is a high risk of adulteration.

Considering the current need for research on ethnomedicine, this work has focused on the evaluation of the antioxidant activity of sample AR along with the pharmacognostic activity. An herbal formulation is considered to be multifunctional as it is usually found in folk medicine that a single plant may be used as a remedy for different ailments. Considering this fact, we have also carried out *in vitro* antidiabetic activity of the sample due to the growing demand of antihyperglycemic substance in the present world where diabetes has affected a large number of the population without any cure to it. Another important reason for carrying out the antidiabetic activity of AR is due to a considerable relation between diabetes and arthritis as people with

diabetes are more likely to have arthritis. A major effect of diabetes includes musculoskeletal changes leading to pain in joint and stiffness with swelling, nodules under the skin, painful shoulders, and feet. Diabetes for a longer period of time may cause joint damage called arthropathy. >26% of adults over 65 are estimated to have diabetes mellitus and diabetes is a key mortality predictor in patients with rheumatoid arthritis as reported by The Centers for Disease Control and Prevention [5,6]. Thus, control of diabetes may also be considered as a preventive measure to decrease the risk of arthritis.

MATERIALS AND METHODS

Plant material

A polyherbal formulation was prepared by mixing the rhizomes of *R. acuminatum*, *A. rivularis,* and whole plant of *V. articulatum* in equal ratio. This formulation was collected from Uttarey, West Sikkim from a herbal practitioner in March 2014. The herbarium specimens were identified and authenticated by Dr. A.P. Das, Professor, Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal. The voucher specimens were deposited at laboratory herbarium; Taxonomy and Environmental Biology Laboratory, University of North Bengal against accession no 06502/Tag no. E.S. 08 for *A. rivularis* Buch.-Ham.ex.D.Don, 06501/Tag no. E.S. 06 for *R. acuminatum* Hook.f. and Thomson, and 09727/Tag no. E.S. 02 for *V. articulatum* Burm.f.

Extraction

Dry powder of AR was extracted through Soxhlet apparatus in ten different solvents successively beginning from non-polar solvent to polar. The extraction was started with hexane (ARHx), heptane (ARHp), benzene (ARBz), ethyl acetate (AREa), chloroform (ARCl), acetone (ARAc), butanol (ARBu), ethanol (AREa), methanol (ARMt), and aqueous (ARAq). These extracts were dried completely and reconstituted in methanol which afterward was stored in the brown bottle for qualitative and quantitative phytochemical estimation along with the antioxidant and antidiabetic activity. Dry powder was used for the pharmacognostic study.

Pharmacognostic study

Organoleptic tests

The powder of AR was tested with our sense organs for color, odour, appearance, texture, and taste [7].

Powder microscopy

The powder of AR was observed under a microscope after staining it with specific stains to observe the fragments of tissues on the basis of standard procedures [8]. The powder was mounted on a glass slide with glycerine and observed under a microscope. To identify the lignified tissues, the powder was stained with alcoholic phloroglucinol solution and conc. HCl. Starch grains were identified with iodine solution (N/10). Sudan III was used for staining any oil globules if present.

Physicochemical analysis

The determination of physicochemical constants such as ash values (total ash, water-soluble ash, and acid insoluble ash), extractive values (water and alcohol), pH values, and moisture content was evaluated [9].

Fluorescence analysis

The chemicals present in plants show fluorescence when it was exposed to ultraviolet (UV) light which can be used as a tool to identify plants even in powder form. AR was treated with some chemical reagents to attain fluorescence. The variation in colors was noted down under visible and UV (254 nm and 365 nm) light [10].

Thin-layer chromatography (TLC)

The protocol of Wagner and Bladt [11] was followed for the identification of active phytoconstituents present in AR. Readymade TLC plates coated with silica gel 60 F-254 purchased from Merck Germany was used. Specific detection reagents were used for the identification of compounds. The developed TLC plates were observed under UV-254 nm and UV-365 nm light. DSLR Nikon camera (D-3200) was used to take pictures and the R_f values of the appeared bands were calculated with the following formula:

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Qualitative estimation of phytochemical

The presence of phytochemicals such as amino acids, tannins, triterpenoids, steroids, alkaloids, and flavonoids present in different solvent extracts of AR was detected according to the standard methods [12-15].

Quantitative estimation of phytochemical

Total phenol content (TPC)

TPC in AR was determined using the Folin–Ciocalteu assay [16]. The extracts of AR (1 ml) were used on which 1 ml 95% ethanol, 5 ml distilled water, and 0.5 ml 50% Folin–Ciocalteu were added. After 5 min of incubation, 1 ml 5% sodium carbonate was added. The mixture was incubated for 1 h and absorbance was taken in UV-spectrophotometer at 725 nm. The phenol content was calculated and expressed as mg gallic acid equivalents (GAE).

Total flavonoids content (TFC)

TFC of AR was calculated by the aluminum chloride colorimetric assay [17]. To 0.5 ml of methanolic sample, 4 ml of distilled water was added followed by 0.3 ml of 5% sodium nitrite. It was incubated for 5 min followed by the addition of 0.3 ml 10% aluminum chloride and incubated again for 6 min. At last, 2 ml of 1 M sodium hydroxide was added and the mixture was diluted with 2.4 ml distilled water. Absorbance was taken at 515 nm. Standard curve of quercetin was prepared for the calculation of TFC of the extracts which were expressed as mg quercetin/g extractive (QE) weight.

Total orthodihydric phenol content (TOPC)

The procedure of Kim *et al.* [18] was followed for the measurement of TOPC of AR. The methanolic extract (0.5 ml) was added in a test tube in which 0.5 ml of 0.05 N HCl and 0.5 ml of Arnow's reagent was added. The mixture was shaken well in a vortex after which 5 ml of distilled water and 1 ml of 1 N sodium hydroxide was added. The appearance of pink color indicated the presence of TOPC, the absorbance of which was taken at 515 nm. The TOPC was expressed in terms of catechol equivalents.

Alkaloid content (AC)

AC was determined by the method based on the reaction between bromocresol green (BCG) and alkaloid of the sample [19]. The methanolic extracts were evaporated and 0.5 ml 2(N) HCl was added filtered and washed with 2 ml chloroform. BCG solution (2.5 ml) and phosphate buffer (2.5 ml, pH-4.7) were added to the chloroform. The mixture was thoroughly mixed by vortexing. The chloroform part (yellow color) was taken for measurement at 470 nm. AC was expressed as solasodine equivalents (SE).

Tannin content (TC)

It was determined in AR by the Folin-Denis reagent [20]. To 0.1 ml of extract, 7.5 ml of distilled water was added followed by 0.5 ml of Folin-Denis reagent and 1 ml of sodium carbonate solution. After incubation of 30 min in room temperature, the absorbance of the blue color mixture was taken at 700 nm. Tannic acid was used for the preparation of standard curve and TC was expressed as tannic acid equivalents.

Steroid content (SC)

Steroid was estimated by the method of Naik and Mishra [21]. To 1 ml of the sample extract, 4 ml of chloroform was added and mixed thoroughly. Chloroform layer (1 ml) was taken and evaporated completely.

Liebermann–Burchard reagent (2 ml) was added to it and mixed well in a vortex. The mixture was kept in the dark for 20 min. The formation of a green colored complex confirmed the presence of steroids in the sample. Absorbance was taken at 640 nm. SC was expressed as SE.

Evaluation of antioxidant activity

1,1-diphenyl-2- picrylhydrazyl scavenging activity (DPPH)

The ability of the extracts to scavenge DPPH free radical was carried out by the method described by Blois [22]. DPPH solution (2 ml) was added to 0.2 ml of methanolic extracts. Absorption was taken at 517 nm. IC_{50} values were calculated from the inhibition percentage against concentration curve. Percentage inhibition was calculated by the following formula:

$$\text{Inhibition percentage} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100$$

2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt scavenging activity

$ABTS^{+}$ solution (2 ml) was added to 1 ml of extract, incubated for 10 min and scavenging activity was measured by taking optical density (OD) values at 734 nm [23]. Inhibition percentage was calculated.

Superoxide (SO) scavenging activity

SO of AR was measured by the reduction of nitro blue tetrazolium (NBT) chloride according to the method of Nishikimi *et al*. [24]. The reaction mixture containing 1 ml sample, 1 ml NBT (312 µM), 1 ml nicotinamide dinucleotide trihydrochloride, and 10 µl phenazine methosulfate was incubated for 30 min under the exposure to fluorescent light. Absorbance was taken at 560 nm. Percentage inhibition was calculated.

Nitric oxide (NO) scavenging activity

Nitrite ions are generated when NO produced by sodium nitroprusside reacts with oxygen. These nitrite ions are detected by Greiss reagent turning the reaction mixture dark pink in color [25]. The scavenging activity of NO is measured at 540 nm. Percentage inhibition was calculated.

Metal chelating (MC) activity

Ferrous ion chelation activity of AR was measured by the method of Dinis *et al*. [26]. In the methanolic extract (0.4 ml), 1.6 ml of methanol was added. After that 0.04 ml of 2 mM ferrous chloride and 0.80 ml of 5 mM ferrozine were added. It was incubated at room temperature for 10 min. Absorbance was taken at 562 nm. Percentage inhibition was calculated.

Ferric reducing antioxidant power (FRAP)

A green colored complex was formed by the reaction among potassium ferricyanide, trichloroacetic acid, and ferric chloride. Increase in the absorbance indicates more antioxidant activity. The OD values were taken at 700 nm [27].

Antidiabetic activity

Alpha-glucosidase inhibiting (AGI) activity

The antidiabetic activity of AR extracts was determined by AGI activity [28]. The methanolic samples were dried completely and reconstituted in distilled water for the assay. Phosphate buffer (6.8 pH), 0.1 ml 3 mM glutathione reduced and 0.1 ml α-glucosidase (1 U/ml) taken in a test tube was incubated for 15 min at 37°C. Extract (0.5 ml) and 0.25 ml *ρ*-NPG were added as a substrate, and it was incubated again for 15 min at 37°C. When the incubation was complete, 4 ml sodium carbonate was added on the reaction mixture. The absorbance was taken at 405 nm. Percentage inhibition was calculated.

Statistical analysis

The experiments were performed with triplicates. MS Excel 2007 (Microsoft, Redmond, WA, USA) was used for determining antioxidant and antidiabetic attributes. The means were compared by Duncan's multiple range test through DSAASTAT software (version 1.002). Values with $p<0.05$ were considered significant. IC_{50} values of antioxidants and antidiabetic activity and their standard error of estimates were done in Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA, USA). Correlation coefficient analysis was done using SPSS (Version 12.00, SPSS Inc., Chicago, IL, USA) and principal component analysis (PCA) was conducted in XLSTAT 2014.

RESULTS

Pharmacognostic study

Organoleptic tests revealed that the powder of AR was brown in color, slightly bitter in taste; texture slightly rough and it had no characteristic odor. Microscopic analysis of the powdered sample showed various tissues such as starch granules, calcium oxalate crystals, xylem vessels, parenchymatous cells, sclereids, and stone cells

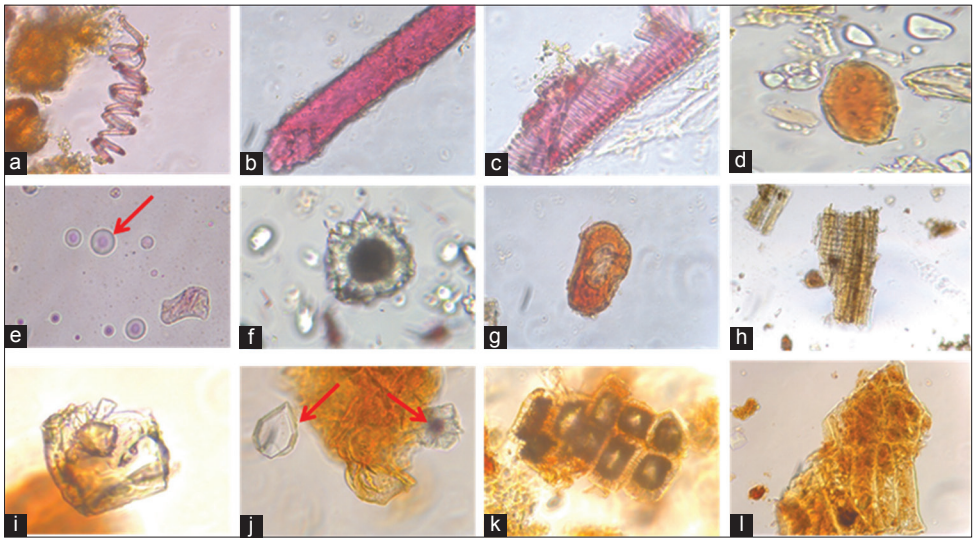


Fig. 1: Powder microscopy of AR. (a) spiral xylem vessels, (b) vessels with pits, (c) scalariform tracheid, (d) oil globule, (e) starch grains with concentric hilum, (f) cluster of calcium oxalate crystals, (g) stone cells, (h) medullary rays with fibers, (i) cuboidal shaped crystal, (j) crystals, (k) sclereids, (l) parenchymatous cells

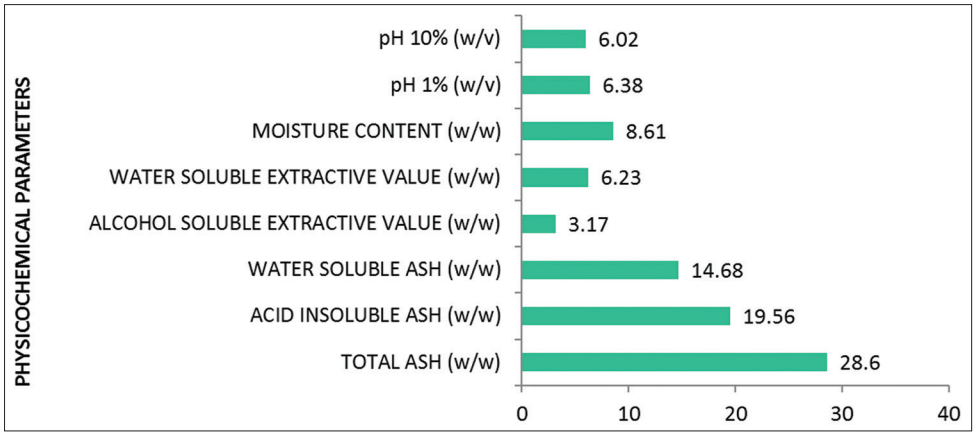


Fig. 2: Physicochemical study of powder polyherbal formulation, AR.

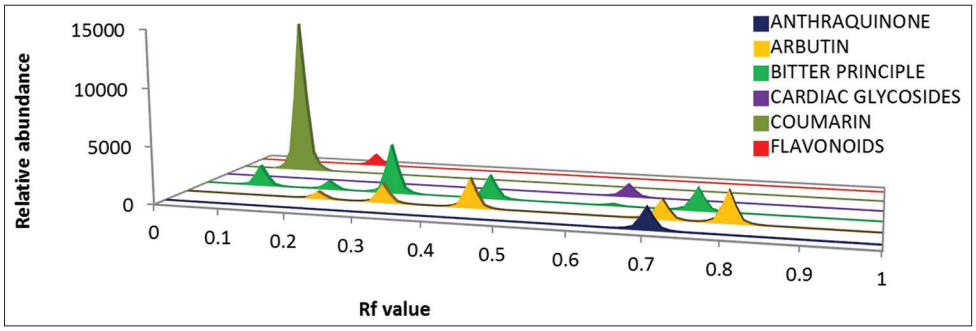


Fig. 3: Relative abundance against Rf values graph of the phytoconstituents identified in thin-layer chromatography plates of AR

Table 1: Fluorescence analysis of AR under visible and UV light. Colors were compared to the standard color chart of RAL (Reichs-Ausschuß für Lieferbedingungen and Gütesicherung)

S. No.	Reagents	Visible	UV-254 nm	UV-365nm
1	Powder+distilled water	Yellow orange	Colorless	Violet blue
2	Powder+50% KOH	Brown red	Colorless	Fir green
3	Powder+benzene	Colorless	Colorless	Signal violet
4	Powder+50% benzene	Lemon yellow	Honey yellow	Broom yellow
5	Powder+chloroform	Lemon yellow	Broom yellow	Ochre yellow
6	Powder+50% chloroform	Broom yellow	Curry yellow	Gray beige
7	Powder+nitric acid (conc.)	Daffodil	Colorless	Colorless
8	Powder+50% nitric acid	Honey yellow	Colorless	Colorless
9	Powder+10% ferric chloride	Curry yellow	Colorless	Colorless
10	Powder+methanol	Honey yellow	Curry yellow	Grey beige
11	Powder+50% methanol	Broom yellow	Brilliant blue	Violet blue
12	Powder+ethanol	Daffodil yellow	Curry yellow	Gray beige
13	Powder+50% ethanol	Broom yellow	Sapphire blue	Ultramarine blue
14	Powder+glacial acetic acid (conc.)	Lemon yellow	Curry yellow	Broom yellow
15	Powder+50% glacial acetic acid	Lemon yellow	Honey yellow	Azure blue
16	Powder+sulfuric acid (conc.)	Copper brown	Granite grey	Azure blue
17	Powder+50% sulfuric acid	Beige	Capri blue	Azure blue

UV: Ultraviolet

(Fig. 1). The physicochemical analysis showed the different parameters of quality control which is illustrated in Fig. 2. Table 1 represents the variation of colors exhibited by AR under UV light after treatment with different chemical reagents. TLC of AR revealed the presence of some phytoconstituents such as anthraquinone, arbutin, flavonoids, cardiac glycosides, coumarins, and bitter principles. The R_f values and relative abundance are calculated and are presented in Table 2 and Fig. 3.

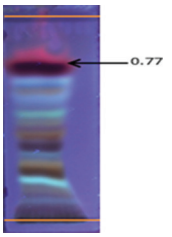
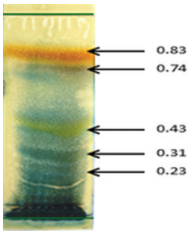
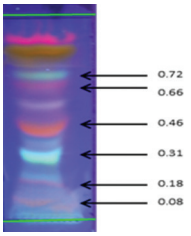
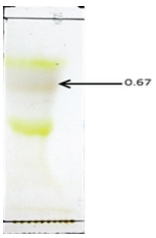
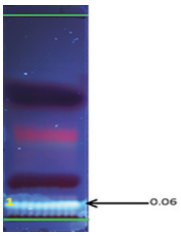
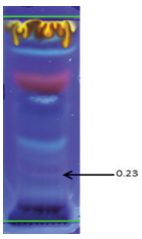
Qualitative phytochemical estimation

Phytochemical screening of different solvent extracts of AR was performed based on standard tests. Some bioactive phytochemicals such as phytosterol, triperpenoids, cardiac glycosides, flavonoids, reducing sugar, resin, and amino acids were present in AR (Table 3). Phytosterol and reducing sugar were present in all the extracts of AR with AREa, ARAc, ARBu, AREt, and ARMt possessing the most concentrated amount of it. Tannins and alkaloids were not detected in the sample while amino acid was present only in ARAq.

Quantitative phytochemical estimation

Most of the phytochemicals present in plants possess medicinal properties which are useful during the treatment of ailments in traditional system of medicine. Even for the development of modern drugs, these phytochemicals are essential. We have, therefore, carried out the quantitative estimation of some phytochemicals such as phenol, flavonoids, orthodihydric phenol, alkaloids, tannin, and steroids which

Table 2: Detection of bioactive phytoconstituents in powder AR by TLC technique

Bioactive phytoconstituent	Solvent system	Detecting reagent	Chromatogram with Rf values
Anthraglycoside (red and yellow band)	Ethyl acetate: methanol: water 100:13.5:10	KOH reagent	
Arbutin (blue and brown bands)	Ethyl acetate: methanol: water 100:13.5:10	Berlin blue	
Bitter principles (blue, red, yellow, brown, green bands)	Ethyl acetate: methanol: water 100:13.5:10	VS reagent (vanillin-sulfuric acid)	
Cardiac glycoside (blue)	Ethyl acetate: methanol: water 100:13.5:10	SbC_{13} reagent (Antimony chloride)	
Coumarin	Toluene: ethyl acetate 93:7	10% ethanolic KOH	
Flavonoids	Formic acid: glacial acetic acid: water 100:11:11:26	NP/PEG reagent	

TLC: Thin-layer chromatography

are known for their health benefits. The results in Table 4 have clearly illustrated that AR is enriched with these phytochemicals especially TPC which was found in highest quantity in ARAc (321.453±0.55 mg GAE/g EW). TFC was concentrated mainly in AREa (44.75±0.07 mg QE/g EW). SC, TC, and TOPC were also detected. Alkaloids were detected only in selective solvent extracts preferably in non-polar solvents such as hexane, heptane, benzene, and ethyl acetate with ARBz exhibiting higher AC (1.80±0.20 mg SE/g EW) than the rest of the solvents. TC was again highest in ARAc with 394.423±0.31 (mg TA/g EW).

***In vitro* antioxidant activity**

Antioxidants are capable of scavenging free radicals and this radical scavenging activity of antioxidants present in AR is illustrated in Table 5. The activity as represented in IC_{50} values with the lowest IC_{50} (highest antioxidant activity) in DPPH and $ABTS^{+}$ scavenging assay was exhibited by ARAc. The acetone extract was also suitable for ferric reducing potentiality (1608.073±0.0002 mg AAE/g EW). MC (3.777±0.62 mg/ml) and SO scavenging potential (0.24±0.002 mg/ml) were found to be highest in ARAq.

Table 3: Qualitative phytochemical screening of AR in different solvent extracts

Phytochemicals	ARHx	ARHp	ARBz	AREa	ARCl	ARAc	ARBu	AREt	ARMt	ARAq
Phytosterol										
Tannin										
Triterpenoids										
Cardiac glycosides										
Glycosides										
Alkaloids										
Flavonoids										
Reducing sugar										
Resins										
Amino acids										
Relative abundance										

Low Medium High

Table 4: Quantitative estimation of phytochemicals of AR

Solvent fractions	TPC (mg GAE/g EW)	TFC (mg QE/g EW)	TOPC (mg CE/g EW)	AC (mg SE/g EW)	TC (mg TA/g EW)	SC (mg SE/g EW)
ARHx	NA	NA	NA	$0.340\pm0.14_{c}$	NA	$0.354\pm0.09_{b}$
ARHp	$20.483\pm0.03_{i}$	$18.95\pm0.02_{d}$	$0.060\pm0.01_{e}$	$0.636\pm0.10_{b}$	$77.190\pm0.01_{h}$	$0.334\pm0.013_{c}$
ARBz	$93.887\pm0.07_{e}$	$33.29\pm0.06_{b}$	$0.269\pm0.06_{d}$	$1.80\pm0.20_{a}$	$180.619\pm0.04_{e}$	$0.852\pm0.06_{a}$
AREa	$152.486\pm0.37_{c}$	$44.75\pm0.07_{a}$	$0.625\pm0.07_{b}$	$0.238\pm0.11_{d}$	$343.231\pm0.\ 29_{b}$	$0.251\pm0.069_{d}$
ARCl	$91.183\pm0.08_{f}$	$13.56\pm0.02_{f}$	$0.056\pm0.02_{e}$	NA	$191.22\pm0.02_{d}$	$0.121\pm0.019_{f}$
ARAc	$321.453\pm0.55_{a}$	$27.19\pm0.15_{c}$	$1.109\pm0.01_{a}$	NA	$394.423\pm0.31_{a}$	$0.073\pm0.037_{h}$
ARBu	$188.636\pm0.52_{b}$	$18.45\pm0.02_{e}$	$1.159\pm0.01_{a}$	NA	$217.096\pm0.21_{c}$	$0.080\pm0.043_{g}$
AREt	$80.781\pm0.51_{g}$	$6.05\pm0.13_{g}$	$0.264\pm0.04_{d}$	NA	$97.544\pm0.09_{f}$	$0.041\pm0.044_{i}$
ARMt	$106.796\pm0.41_{d}$	$0.12\pm0.04_{i}$	$0.488\pm0.06_{c}$	NA	$89.822\pm0.14_{g}$	$0.145\pm0.044_{e}$
ARAq	$64.539\pm0.53_{h}$	$4.6\pm0.06_{h}$	$0.284\pm0.04_{d}$	NA	$6.835\pm0.02_{i}$	$0.032\pm0.055_{j}$

Values with different letters (a, b, c, d, e, f, g, h, i and j) are significantly ($p<0.05$) different from each other by Duncan's multiple range test (DMRT), NA: Not applicable, TPC: Total phenol content, TFC: Total flavonoids content, QE: Quercetin/g extractive, TOPC: Total orthodihydric phenol content, AC: Alkaloid content, TC: Tannin content, SC: Steroid content, SE: Solasodine equivalents, GAE: Gallic acid equivalents

Table 5: *In vitro* antioxidant activity of AR extracts

Solvent fractions	DPPH IC_{50} (mg/ml)	ABTS+IC_{50} (mg/ml)	SO IC_{50} (mg/ml)	NO IC_{50} (mg/ml)	MC IC_{50} (mg/ml)	FRAP (mg AAE/g EW)
ARHx	$3.087\pm0.22_{g}$	$1.813\pm0.33_{e}$	$2.352\pm0.06_{f}$	NA	NA	$50.101\pm0.36_{h}$
ARHp	$2.501\pm0.02_{f}$	$0.684\pm0.01_{d}$	NA	NA	NA	NA
ARBz	$2.615\pm0.07_{f}$	$0.372\pm0.08_{c}$	$0.461\pm0.04_{bc}$	$2.139\pm0.02_{b}$	NA	NA
AREa	$0.328\pm0.03_{c}$	$0.079\pm0.03_{a}$	$0.613\pm0.19_{c}$	NA	NA	$343.706\pm0.01_{d}$
ARCl	$0.986\pm0.02_{e}$	$0.277\pm0.03_{bc}$	$1.883\pm0.04_{e}$	NA	NA	$294.514\pm0.06_{e}$
ARAc	$0.085\pm0.004_{a}$	$0.043\pm0.01_{a}$	$0.904\pm0.02_{d}$	$2.479\pm0.02_{b}$	NA	$1608.07\pm0.001_{a}$
ARBu	$0.145\pm0.01_{ab}$	$0.084\pm0.04_{a}$	$0.327\pm0.08_{b}$	$5.381\pm0.44_{d}$	$16.681\pm0.55_{c}$	$726.834\pm0.01_{c}$
AREt	$0.331\pm0.05_{c}$	$0.127\pm0.02_{a}$	$1.005\pm0.03_{d}$	$7.399\pm0.38_{e}$	$15.162\pm0.01_{b}$	$346.946\pm0.15_{c}$
ARMt	$0.231\pm0.01_{bc}$	$0.115\pm0.01_{ab}$	$0.402\pm0.11_{b}$	$3.699\pm0.42_{c}$	$3.974\pm0.01_{b}$	$130.082\pm0.07_{g}$
ARAq	$0.588\pm0.07_{d}$	$0.138\pm0.02_{ab}$	$0.24\pm0.002_{a}$	NA	$3.777\pm0.61_{a}$	$167.153\pm0.01_{f}$
STD	$0.11\pm0.01_{ab}$ (AA)	$0.28\pm0.001_{bc}$ (AA)	$0.03\pm0.01_{a}$ (AA)	$0.03\pm0.002_{a}$ (CC)	$0.01\pm0.001_{a}$ (BHT)	

AA: Ascorbic acid; CC: Curcumin; BHT: Butylated hydroxytoluene, Values with different letters (a, b, c, d, e, f, g and h) are significantly ($p<0.05$) different from each other by Duncan's multiple range test (DMRT), NA: Not applicable, STD: Standard, DPPH: 1,1-diphenyl-2- picrylhydrazyl scavenging activity, SO: Superoxide, NO: Nitric oxide, MC: Metal chelating, FRAP: Ferric reducing antioxidant power, ABTS: 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)

Antidiabetic activity

The antidiabetic activity of AR extracts was evaluated by the inhibition of the α-glucosidase enzyme. Some of the extracts showed potential antidiabetic activity when compared with standard drug metformin (Fig. 4). Inhibition of enzyme by some extracts of AR were remarkable while some extracts showed no inhibition at all. Some of the extracts showing good inhibition were ARAc, AREa, and ARBu with ARAc exhibiting highest antidiabetic activity (0.26±0.003 mg/ml). The IC_{50} value of metformin (standard) was 0.175±0.03 mg/ml.

DISCUSSION

Folk medicine is one of the oldest forms of health-care system that has proved its efficacy to the test of time. The growing interest in search of natural source of medicine has opened a scope for folk medicine to be recognized globally. The herbal medicine is usually found in a dried form where the parts of plants are either powder or are twisted and rolled which makes it impossible to be identified. Therefore, this work is an attempt to study an ethnomedicine (AR) which is a combination of three plants/parts which is traditionally used for the treatment of arthritic pain in a village of West Sikkim. First, we have tried to observe some diagnostic and diabetes characteristics of AR which could be used to establish a standard for this formulation for authenticity and quality control which could be helpful for preparing herbal monographs as emphasized by the WHO. Out of many tissues observed in powder microscopy, AR powder contained few prominent tissues such as a large number of starch granules with concentric hilum, different shapes of crystals, and spiral xylem vessels. The physicochemical study is another very important parameter for quality control. Total ash values are measured for the detection of adulterants such as stone, silica, and some other earthy materials [29]. Acid-insoluble ash represents the

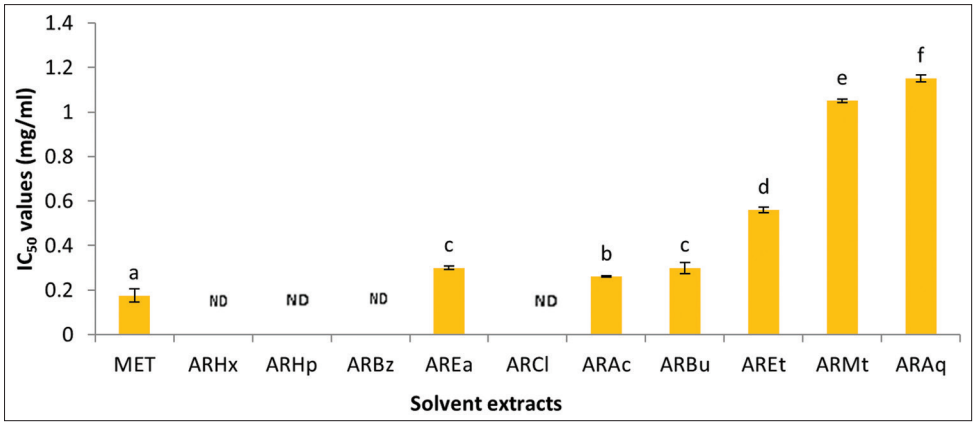


Fig. 4: Alpha-glucosidase inhibiting activity of AR compared with standard drug metformin (MET). Values with different letters (a, b, c, d, e and f) are significantly (p<0.05) different from each other by Duncan's multiple range test; ND: Not detected

presence of these materials which was found to be 19.56% (w/w) and water soluble ash was 14.68% (w/w) which indicates that sample AR should be taken more care of while handling and preparation as the amount of acid insoluble ash should be as low as possible. The moisture content of AR was 8.61% (w/w) which was within the limit of the recommended range of not exceeding 14% and thus it can be stored for a longer period of time, and there is a less probability for microbial growth. The water-soluble extractive value was higher (6.23% w/w) than alcohol soluble extractive (3.17% w/w) which suggests that water is suitable to achieve the better amount of extracts than alcohol from AR. The pH values are almost alkaline which is >6 make the formulation safer for consumption. Fluorescence is a phenomenon which is exhibited by various phytochemicals. Each chemical has a specific color which if not attained directly may reveal colors after treating with particular chemical reagents [30,31]. Moreover, this parameter is regarded as one of the important methods for assessing the quality of powder drugs. The variation of colors from AR under visible and UV light (254 nm and 365 nm) is presented in Table 1. The variation in colors may also indicate the diversity of chemical compounds present in the sample [32]. Another parameter we have selected for standardization of AR is through TLC technique (Table 2 and Fig. 3). It is usually performed to develop a basic idea for the presence of some active phytoconstituents in the sample. The R_f values of the bands representing a compound are specific if carried out in the same solvent system and detection reagents. Thus, it can be useful for the quality check of the same formulation in the future. The detail of the solvent system and detection reagents along with the developed chromatogram images is summarized in Table 2. TLC has not only provided the qualitative data of the phytochemicals but also offer the quantitative profile of the phytoconstituents present with relative abundance as given in Fig. 3. Several bands of arbutin and bitter principles were observed, but coumarin showed the highest relative abundance which indicates the quantity of the particular phytoconstituent. Hence, TLC serves as an important tool for the preliminary evaluation of a crude drug[11]. Since the therapeutic activity of any medicinal plants is attributed to the various secondary metabolites present in it, thus we have carried out a qualitative screening of phytochemicals present in AR through standard tests. As the nature of phytochemical constituents present in plants is diverse, we cannot say that a particular solvent is suitable to extract all the bioactive compounds [33]. To fulfill the range of polarity we have extracted AR in 10 different solvents from non-polar to polar as mentioned earlier. The results have also indicated that solvents play a crucial role in the extraction of compounds from plants. Table 3 justifies this point where it is clear that ethyl acetate, acetone, butanol, and methanol have concentrated the amount of phytochemicals while in case of amino acid, it was observed that only aqueous sample gave a positive result. Similarly, antioxidant activity was also highly influenced by solvent extraction methods. TPC was highest in acetone extract of AR which is supported by previous studies also [34]. AC was not detected in TLC and qualitative test with Dragendorff's reagent, but it was quantitatively observed particularly in polar solvents up to ethyl acetate. It may also indicate that for a particular phytochemical, a single test is not efficient. More than one test should be performed for a single phytochemical test. The analysis of physicochemical and phytochemical attributes mainly helps to evaluate the phytoconstituents present in a particular extract and to find the solubility of a particular constituent in a specific solvent [35]. Plants produce antioxidants in the form of secondary metabolites such as phenolics and flavonoids to cope up with the stress caused by ROS [36]. The activity of these antioxidants of AR was evaluated through free radical scavenging activity where it was observed that some of the extracts of AR showed better DPPH and $ABTS^+$ scavenging activity than standard ascorbic acid. Here again, acetone extract was found to be the best when compared to other extracts as it also showed the highest reducing powder. In case of reducing power, it was observed that the range of activity was aqueous<methanol<ethanol<acetone but after that, it again decreased as it proceeded further toward non-polar solvents. This may be due to the fact that the nature of compounds responsible for reducing power ranges in moderately polar solvents.

The antidiabetic activity was evaluated by hindering the absorption of glucose through inhibition of the carbohydrate hydrolyzing enzyme, α-glucosidase, in the digestive tract. Again acetone extract exhibited the highest inhibition of α -glucosidase enzyme (Fig. 4) which was quite close to standard drug metformin. It shows that AR has a potential antidiabetic activity which could be further explored with broader parameters of toxicity and *in vivo* models.

Correlation

The Pearson correlation was analyzed to carry out the relationship amongst antioxidant activity, antidiabetic activity with its phytochemicals content (Table 6). It showed a significant positive correlation between DPPH and $ABTS^+$ scavenging activity ($p<0.01$) and a significant negative correlation for DPPH to TPC and TOPC, respectively. Since this indicates that the increase in TPC and TOPC will decrease the IC_{50} values of DPPH, it is clear that the group of phenols present in AR is responsible for the scavenging activity of DPPH and $ABTS^+$. Several such reports are available where phenols were considered having antioxidant activity with the scavenging potentiality of free radicals [37,38]. Antioxidant of phenolics is often attributed to its redox potentiality allowing them to act as hydrogen donors, singlet oxygen scavengers, and redox agents [39]. The mechanism of the Folin–Ciocalteu method is based on the reducing properties of phenolic compounds. This is evident in our study also where a strong positive correlation was observed between TPC, TOPC, and reducing power (FRAP) with significant $p<0.01$. TC showed a significant positive correlation with alkaloid and steroid content, but none of these showed any correlation with the antioxidant activity that we had carried out in our study. It is possible that the presence of these compounds was not particularly responsible for the antioxidant activity of AR. The antidiabetic activity was prominent in this study, but none of the phytochemicals we

Table 6: Pearson correlation matrix of antioxidant and antidiabetic activity of AR extracts

	DPPH	ABTS	SO	NO	MC	FRAP	TPC	TFC	TOPC	AC	TC	SC
ABTS	0.819**											
SO	0.291	0.604										
NO	−0.467	−0.398	−0.174									
MC	−0.474	−0.330	−0.206	0.855**								
FRAP	−0.536	−0.375	−0.003	0.175	0.055							
TPC	−0.659*	−0.603	−0.178	0.291	0.112	0.934**						
TFC	−0.112	−0.366	−0.300	−0.151	−0.253	0.409	0.562					
TOPC	−0.671*	−0.555	−0.347	0.422	0.379	0.816**	0.897**	0.469				
AC	0.709*	0.363	−0.302	−0.343	−0.356	−0.386	−0.442	0.152	−0.422			
TC	0.577	0.120	−0.014	−0.379	−0.435	−0.315	−0.314	0.182	−0.493	0.690*		
SC	0.615	0.169	0.008	−0.397	−0.431	−0.351	−0.361	0.154	−0.527	0.703*	0.998**	
AGI	−0.170	−0.167	−0.305	−0.239	0.027	−0.135	−0.186	−0.296	−0.127	−0.201	−0.251	−0.236

**Correlation is significant at the 0.01 level (2-tailed), *Correlation is significant at the 0.05 level (2-tailed), DPPH: 1,1-diphenyl-2- picrylhydrazyl scavenging activity, APTS: 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), SO: Superoxide, NO: Nitric oxide, MC: Metal chelating, FRAP: Ferric reducing antioxidant power, TPC: Total phenol content, TFC: Total flavonoids content, TOPC: Total orthodihydric phenol content, AC: Alkaloid content, TC: Tannin content, SC: Steroid content, AGI: Alpha-glucosidase inhibiting

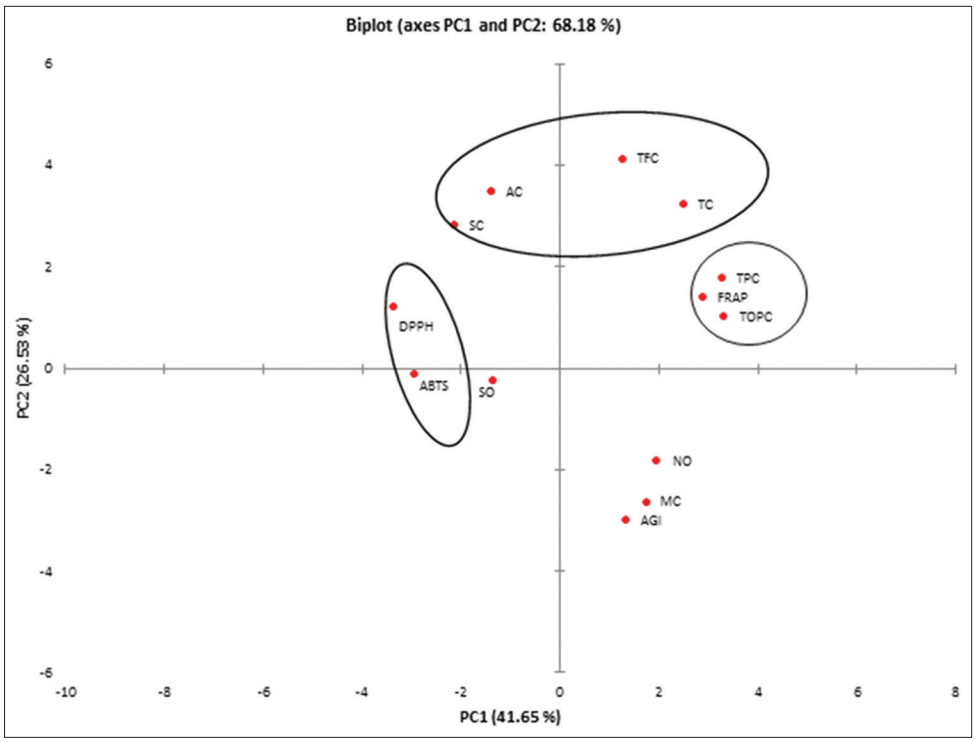


Fig. 5: Principal component analysis of antioxidant, antidiabetic activity, and quantitative phytochemical estimation of AR

had tested showed any correlation for this activity. It, therefore, pointed toward the diverse and complex nature of phytochemicals which are present in plants performing their specific functions. Perhaps AR contains other active phytoconstituents compounds which were responsible for the antidiabetic activity as reported here.

PCA

PCA is the simplest of the multivariate statistical analyses through which the primary pattern of the multiple variables in an experiment can be visualized [40]. In this study, PCA was analyzed to observe the overall relationship of antioxidants, antidiabetic activities, and phytochemicals detected quantitatively in AR. First and second principal components (PC1 and PC2) accounted for 41.65% and 26.53 % of the variance, respectively, totalizing 68.18% (Fig. 5). The loading plot displays the association of different variables with each other. The loading plot showed that TPC, TOPC, and FRAP were loaded positively on PC1 with squared cosine values of 0.7712, 0.7949, and 0.6027, respectively. TFC, AC, TC, and SC were loaded positively on PC2 with squared cosine values of 0.7921, 0.5624, 0.4831, and 0.3723, respectively. The cluster of DPPH and ABTS lies in the opposite direction to the cluster of TPC, TOPC, and FRAP which indicates that they are negatively correlated with each other. It is also supported by the above result of Pearson correlation analysis. Wang and Hu [41] performed PCA with antioxidant attributes of mulberry fruits where similar clusters were formed with TPC, ferric reducing power and oxygen radical absorbance capacity being positively loaded on PC1 [42].

CONCLUSION

Our study has attempted to establish a standard for an ethnomedicine AR. This can be used as a basic reference of this powder formulation which could be easily optimized, cheaper, and authentic and reproducible. Further standardization techniques might also be suggested which can be done with sophisticated analytical instruments such as gas chromatography-mass spectrometry, Fourier transform infrared, and nuclear magnetic resonance for the comprehensive study, but the cost of analysis would be much higher. We were also able to explore its antidiabetic activity along with the influence of solvents on the extraction compounds specifically for a particular activity. AR

showed potential antioxidant capacity along with antidiabetic activity which could further be explored in an animal model.

ACKNOWLEDGMENT

We are grateful to the UGC for providing financial support under the scheme of Rajiv Gandhi National Fellowship. We would also like to acknowledge the Forests, Env. and W/L Management Department Government of Sikkim, Gangtok, for providing the permit for survey in West Sikkim. We are extremely thankful toward Mr. Nar Bahadur Subba, an herbal practitioner for providing us the sample to perform this work.

AUTHORS' CONTRIBUTIONS

Palash Mandal conceived and designed the experiments, participated in the editing of the manuscript.

Arunika Subba performed the experiments and participated in the analysis of data and writing of the manuscript.

CONFLICTS OF INTEREST

Authors declare no conflicts of interest.

REFERENCES

1. Huang DH, Chen C, Lin C, Lin Y. Antioxidant and antiproliferative activities of water spinach (*Ipomoea aquatica* Forsk.) constituents. Bot Bull Acad Sci 2005;46:99-106.
2. Yildrim A, Oktay M, Bilaloglu V. The antioxidant activity of leaves of *Cydonia vulgaris*. Turk J Med Sci 2001;31:23-27.
3. Khilifi S, El-Hachimi Y, Khalil A, Es-Safi N, Belahyan A, Tellal R, *et al*. *In vitro* antioxidant properties of *Salvia verbenaca* L. hydromethanolic extract. Ind J Pharmacol 2006;38:276-80.
4. Akinmoladun AC, Ibukun EO, Afor E, Akinrinlola BL, Onibon TR, Akinboboye AO, *et al*. Chemical constituents and antioxidant activity of *Alstonia boonei*. Afr J Biotechnol 2007;6:1197-201.
5. Innala L, Möller B, Ljung L, Magnusson S, Smedby T, Södergren A, *et al*. Cardiovascular events in early RA are a result of inflammatory burden and traditional risk factors: A five year prospective study. Arthritis Res Ther 2011;13:R131.
6. Michaud K, Wolfe F. Comorbidities in rheumatoid arthritis. Best Pract Res Clin Rheumatol 2007;21:885-906.
7. Kokate CK, Purohit AP, Gokhale SB. Text Book of Pharmacognosy. 18th ed. Pune: Nirali Prakashan; 2002.
8. Lala PK. Practical Pharmacognosy. 1st ed. Calcutta: Lina; 1981.
9. Harborne JB. Phytochemical Methods-a Guide to Modern Techniques of Plant Analysis. Berlin: Springer Verlag; 2005.
10. Chase CR, Pratt R. Fluorescence of powdered vegetable drugs with particular reference to development of a system of identification. J Pharm Sci 1949;38:324-31.
11. Wagner H, Bladt S. Plant Drug Analysis: A Thin Layer Chromatography Atlas. 2nd ed. Berlin: Springer Verlag; 2009.
12. Kumar A, Ilavarasan R, Jayachandan T, Decaraman M, Aravindhan P, Padmanabhan N. Phytochemical investigation on a tropical plant, *Syzygium cumini* from Kattuppalayam, Erode District, Tamil Nadu, South India. Pak J Nutr 2009;8:83-5.
13. Ngbede J, Yakubu RA, Nyam DA. Phytochemical screening for active compound in *Canaruim schweinfurthii* (Atile) leaves from Ios North, Plateau state, Nigeria. Res J Biol Sci 2008;3:1076-8.
14. Ibrahim HA, Ibrahim H. Phytochemical screening and toxicity evaluation on the leaves of *Argemone mexicana* Linn. (Papaveraceae). Int J Pure Appl Sci Technol 2009;3:39-43.
15. Trease GE, Evans WC. Pharmacognosy. 13th ed. London: Bailliere Tuidal; 1989. p. 799-80.
16. Singleton, VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. Am J Enol Vitic 1965;16:144-58.
17. Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem 1999;64:555-9.
18. Kim DO, Jeong SW, Lee CY. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. Food Chem 2003;81:321-6.
19. Ajanal M, Gundkalle MB, Nayak SU. Estimation of total alkaloid in chitrakadivati by UV-spectrophotometer. Anc Sci Life 2012;31:198-201.
20. Thimmaiah SR. Standard Methods of Biochemical Analysis Chapter Phenolics. New Delhi India: Kalyani Publishers; 1999. p. 296-7.
21. Naik PD, Mishra RL. Quantitaive analysis of secondary metabolites of *Withania somnifera* and *Datura stramonium*. Int J Sci Res 2015;4:300-3.
22. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 1958;181:1199-200.
23. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C, *et al*. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999;26:1231-7.
24. Nishikimi M, Appaji N, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun 1972;46:849-54.
25. Marcocci L, Packer L, Droy-Lefaix MT, Sekaki A, Gardès-Albert M. Antioxidant action of *Ginkgo biloba* extract EGb 761. Methods Enzymol 1994;234:462-75.
26. Dinis TC, Maderia VM, Almeida LM. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch Biochem Biophys 1994;315:161-9.
27. Oyaizu M. Studies on products of browning reactions: Antioxidative activities of products of browning reaction prepared from glucoamine. Jpn J Nutr 1986;4:307-15.
28. Shai LJ, Magano SR, Lebelo SL, Mogale AM. Inhibitory effects of five medicinal plants on rat alpha-glucosidase: Comparison with their effects on yeast alpha-glucosidase. J Med Plant Res 2011;5:2863-7.
29. Musa KY, Katsayal AU, Ahmed A, Mohammad A, Danmalam Z. Pharmacognostic investigation of the leaves of gisekia pharmacioides. Afr J Biotechnol 2006;5:956-7.
30. Kokoski CJ, Kokoski RJ, SLAMA FJ. Fluorescence of powdered vegetable drugs under ultraviolet radiation. J Am Pharm Assoc Am Pharm Assoc 1958;47:715-7.
31. Deb J, Dash GK. Pharmacognostical studies on stem bark of *Acacia ferruginea* DC. Pharmacia Lettre 2014;6:61-6.
32. Ansari SH. Essential of Pharmacognosy. 1st ed. New Delhi: Birla Publications Pvt. Ltd.; 2007.
33. Ordonez AA, Gomez JD, Vattuone MA, Isla MI. Antioxidant activities of *Sechium edule* (Jacq). Food Chem 2006;97:452-8.
34. Wintola OA, Afolayan AJ. Phytochemical constituents and antioxidant activities of the whole leaf extract of *Aloe ferox* mill. Pharmacogn Mag 2011;7:325-33.
35. Duraiswamy B, Singanan M, Varatarajan V. Physicochemical, phytochemicals and antioxidant evaluation of *Guazuma ulmifolia* fruit. Int J Pharm Pharm Sci 2018;10:87-91.
36. Nwaleaego NL, Chibuike OK, Chukwugekwu EM, Marylyn AC, Ngozi EI, Chukwunonye ER. *In vitro* antioxidant and free radical scavenging potential of methanolic extracts of *Uvaria chamae* leaves and roots. Int J Pharm Pharm Sci 2019;11:67-71.
37. Rahman MM, Khan FE, Das R, Hossain MA. Antioxidant activity and total phenolic content of some indigenous fruits of Bangladesh. Int Food J Res 2016;23:2399-404.
38. Miliauskas G, Venskutonis PR, van Beek TA. Screening of radical scavenging activity of some medicinal plants and aromatic plant extract. Food Chem 2004;85:231-7.
39. Macheix JJ, Fleuriet A. Phenolic acids in fruits. In: Rice-Evans CA, Packer L, editors. Flavonoids in Health and Disease. New York: Marcel Dekker Inc.; 1998.
40. Dey P, Chaudhuri TK. Antioxidant capacity of *N. indicum*: A correlation study using principal component analysis and multivariate statistical approach. Int J Pharma Pharm Sci 2013;5:931-7.
41. Wang RJ, Hu ML. Antioxidant capacities of fruit extracts of five mulberry genotypes with different assays and principal components analysis. Int J Food Prop 2011;14:1-8.
42. Ghosal M, Mandal P. Phytochemical screening and antioxidant activities of two selected 'Bihi' fruits used as vegetables in Darjeeling Himalaya. Int J Pharm Pharm Sci 2012;4:567-74.

Pharmacogn. Res.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcogres.com | www.phcog.net

ORIGINAL ARTICLE

Alpha Glucosidase Inhibiting Activity and *in vivo* Antidiabetic Activity of *Fraxinus floribunda* Bark in Streptozotocin-Induced Diabetic Rats

Arunika Subba, Ram Kumar Sahu[1], Shiv Bhardwaj[2], Palash Mandal

Department of Botany, Plant Physiology and Pharmacognosy Research Laboratory, University of North Bengal, Siliguri, West Bengal, [1]Department of Pharmacy, Pt. Deendayal Upadhyay Memorial Health Sciences and Ayush University of Chhattisgarh, Naya Raipur, [2]Department of Pharmacology, Columbia Institute of Pharmacy, Tekari, Raipur, Chhattisgarh, India

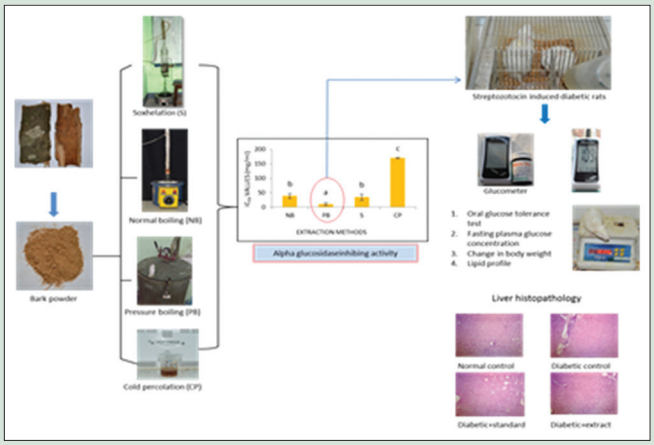
ABSTRACT

Background: Diabetes mellitus is a serious health problem being the third largest cause of death worldwide. Natural sources of antidiabetic agents are of high demand due to side effects of modern drugs. Bark of *Fraxinus floribunda* (FF) is conventionally used in Sikkim to treat diabetes, but there is not a single documented report on the same. **Objective:** The aim of this study is to evaluate *in vitro* and *in vivo* antidiabetic activity of FF bark. **Materials and Methods:** FF bark was extracted through four methods, namely normal boiling, pressure boiling (PB), soxhlet, and cold percolation to be subjected to α-glucosidase inhibiting assay. The extract showing the highest *in vitro* antidiabetic activity was selected for *in vivo* antidiabetic activity. **Results:** Extract from PB showed the highest antidiabetic activity (10.25 ± 4.56 mg/ml FWT); thus, it was selected for antidiabetic property in animal model. The extracts (200 and 400 mg/kg) significantly ($P < 0.05$) reduced plasma glucose concentration in streptozotocin-induced diabetic rats. Glibenclamide (0.50 mg/kg) was used as standard. Decrease in bodyweight during diabetes was significantly controlled by the extract which was comparable with the standard at the same concentration. Changes in lipid profile (total cholesterol, triglycerides, high-density lipoprotein, and low-density lipoprotein) of the diabetic rats were also maintained almost to the level of normal rats by the extracts. Histopathology of liver sections of diabetic rats showed damage in the hepatic architecture (swelling of sinusoids, vacuolization of cytoplasm, and inflammation of the central vein) which was controlled by the extracts. **Conclusion:** This study agrees with the traditional use of FF bark as an antidiabetic agent.

Key words: Antidiabetic, *Fraxinus floribunda,* lipid profile, liver histopathology, streptozotocin, α-glucosidase

SUMMARY

- The bark of *Fraxinus floribunda* Wallich is traditionally used for the treatment of high blood sugar/diabetes by the herbal practitioners of Sankhu, a village in West Sikkim, India. Since there was not any scientific report available on this plant, this study has been done to evaluate *in vitro* and *in vivo* antidiabetic activity on the bark of this plant. The bark was extracted through four different methods (boiling, soxhletion, pressure boiling (PB) and cold percolation) to observe the influence on bioactivity due to the variation in extraction methods. Alpha-glucosidase inhibiting the activity of the extracts was performed the result of which showed the extracts obtained through PB exhibited the highest antidiabetic activity. It further helped to select the extract for investing antidiabetic activity in animal model. Streptozotocin (STZ)-induced rats were used for the same. The parameters taken for *in vivo* experiments were change in body weight, oral glucose tolerance test, fasting plasma glucose concentration, lipid profile, and liver histopathology. The results showed the control in loss of bodyweight, control in increase of glucose level and improved lipid profile of STZ-induced diabetic rats by 200 and 400 mg/kg extracts. The liver histopathology also revealed improvement in architectural changes noticed in diabetic rats by the same concentration of extracts. This study supports the use of the plant as antidiabetic agent in traditional medicine and also suggests further investigation in purifying the bioactive phytochemical responsible for the antidiabetic activity.



Abbreviations Used: FF: *Fraxinus floribunda*; DM: Diabetes mellitus; T2DM: Type 2 diabetes mellitus; PPHG: Postprandial hyperglycemia; NB: Normal boiling; PB: Pressure boiling; S: Soxhelation; CP: Cold percolation; OGTT: Oral glucose tolerance test; NIDDM: Noninsulin-dependent Diabetes mellitus; STZ: Streptozotocin; TCL: Total cholesterol; TGL: Triglycerides; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; DMRT: Duncan multiple range test.

Correspondence:
Dr. Palash Mandal,
Department of Botany, Plant Physiology and Pharmacognosy Research Laboratory, University of North Bengal, Raja Rammohunpur, Siliguri - 734 013, West Bengal, India.
E-mail: pmandalbotppprl@nbu.ac.in
DOI: 10.4103/pr.pr_32_19

Access this article online
Website: www.phcogres.com
Quick Response Code:

INTRODUCTION

Diabetes mellitus (DM) simply known as diabetes is a condition of disordered metabolism characterized by abnormally high level of blood sugar (hyperglycemia) in the body which mainly occurred either due to hereditary causes or sedentary lifestyle.[1] It is also represented by lipidemia and oxidative stress; it affects the patients to long-term complications causing damage in the eyes, kidneys, nerves, skin, and blood vessels.[2] Other than hyperglycemia, it is

Cite this article as: Subba A, Sahu RK, Bhardwaj S, Mandal P. Alpha glucosidase inhibiting activity and *in vivo* antidiabetic activity of *Fraxinus floribunda* bark in streptozotocin-induced diabetic rats. Phcog Res 2019;11:273-8.

characterized by disturbances in the metabolism of carbohydrate, protein, and fat. Hyperlipidemia is another factor of DM which could develop micro and macrovascular complications of diabetes resulting mostly into morbidity or death.[3] Type 2 diabetes mellitus is the most common diabetes causing > 80% of the total cases of diabetes.[4] Insulin is the hormone released by β-cells of the pancreas, which is responsible for glucose homeostasis.[5] Stimulation of hepatocytes, myocytes, and adipocytes by insulin leads to the uptake of glucose from the circulatory system. Glucose can either be used in glycolysis or stored inside muscle or liver cells in the form of glycogen. If insulin is inappropriately utilized, it results in insulin resistance or the inability of cells to respond to normal circulating insulin levels, thus leading to the incidence of the disease. Increased level of postprandial hyperglycemia (PPHG) is one of the highest risk factors.[6] PPHG is elevated due to the action of α-glucosidase and α-amylase enzymes. Inhibition of these enzymes can manage PPHG in diabetic patients. Inhibition of α-glucosidase enzyme activity can reduce disaccharide hydrolysis, which has useful effects on glycemic index control in diabetic patients.[7] Several α-glucosidase inhibitors have been isolated from medicinal plants for the development of new drugs with increased potency and lower adverse effects than the existing drugs.[8,9]

Recently, diabetes is one of the most prevalent diseases in the world which is rapidly increasing worldwide. According to the WHO, the occurrence of diabetes might increase by 35% in the near future. Currently, over 150 million populations in the world are affected by diabetes, which is likely to increase over 300 million or more by the year 2025. In India, the number of diabetic people will increase from 15 million in 1995 to 57 million in the year 2025, which is considered to be the highest number of diabetics in the world.[10] Despite considerable development in the treatment of diabetes by oral anti-hyperglycemic agents, search for newer drugs is still persisting due to therapeutic limitations of existing synthetic drugs with some seriously harmful side effects after prolonged usage.[11,12] Therefore, proper management of diabetes without any drug-mediated side effects is still being a big challenge.[13] However, the researchers are working to find safer, more efficient, and less expensive remedy for diabetes. Numerous medicinal plants have been reported to have antidiabetic property, and they have been used immensely as antidiabetic and antihyperlipidemic remedies.[14]

Fraxinus floribunda Wallich (FF) belonging to family Oleaceae is a plant usually found in the Eastern Himalayas of India, particularly in Khasi Hills and Sikkim. The bark of this plant is conventionally used for the treatment of diabetes in the villages of West district of Sikkim. Earlier reports have suggested the presence of antioxidant, hepatoprotective, and anti-inflammatory activity on the aqueous extract of the bark of FF.[15] Although it is popularly used as an antidiabetic agent in the traditional system, but there was not a single report on the scientific study of this plant. Thus, this work has been carried out to determine the antidiabetic activity of the bark of FF, which might also validate the use of this plant in traditional system of medicine.

MATERIALS AND METHODS

Plant material

The fresh bark of FF was collected from a village called Sankhu in Dentam constituency of West Sikkim. The herbarium of the plant was submitted in the NBU-Herbarium which was identified by Dr. A.P. Das (former Professor), Plant Taxonomy and Environmental Biology, Department of Botany, University of North Bengal. A voucher specimen, accession no. 9632/Tag no E.S.03 was preserved for future reference.

Animals

Wister albino rats (150–250 g) were used in this study. They were obtained from the Animal House of Columbia Institute of Pharmacy, Raipur. They were housed in large propylene cage and kept at 22°C ± 2°C in 12 h dark light cycle of 12:12 h, relative humidity 55%–65%. The animals were fed with pellet food and water *ad libitum*. The experiments were approved by the Institutional Animal Ethics Committee of Columbia Institute of Pharmacy, Raipur, India (Regd. No. 1321/PO/ReBi/S/10/CPCSEA). All the animals were acclimatized for at least 1 week before the experimental session.

Preparation of plant sample

The fresh bark of FF was sun-dried until the moisture was gone. The dried bark was ground into fine powder and further extracted through four different methods, namely normal boiling, autoclave under pressure boiling (PB), soxhlation in a Soxhlet apparatus (S) and cold percolation below – 4°C (CP). All the extracts were measured and a required volume was made up and the extracts were stored in a refrigerator.

Inhibition of α-glucosidase enzyme

A 0.2 M phosphate saline buffer (pH 6.8) was prepared to dissolve α-glucosidase enzyme in concentration of 0.2 U/ml, 3 mM glutathione (reduced), and 10 mM p-NPG (substrate). To 2.5 ml of buffer, 0.1 ml gluthathione and 0.1 ml enzyme were added and incubated for 15 min at 37°C. After incubation, 0.5 ml of inhibitor (extract) was added except in control followed by 0.25 ml p-NPG. The mixture was incubated for 15 min at room temperature and the reaction was inhibited by the addition of 4 ml sodium carbonate.[16] The absorbance of yellow color of reaction mixture was taken at 405 nm.

The percentage inhibition was calculated as:

$$\text{Percentage inhibition} = \left(\frac{\text{Control} - \text{Test sample}}{\text{Control}}\right) \times 100$$

Oral glucose tolerance test

The oral glucose tolerance test (OGTT) was performed in overnight fasted (18 h) normal rats. Rats divided into three groups (n = 6) were administered drinking water, the second group with aqueous extract of 200 mg/kg, and the third group with 400 mg/kg aqueous extract. Glucose (0.5 mg/kg) was fed 30 min prior to the administration of extracts. Blood was withdrawn from the retro-orbital sinus at 30 and 90 min of extract administration, and the plasma obtained after centrifugation at 3000 rpm was estimated for fasting plasma glucose levels using a glucose oxidase-peroxidase glucose estimation kit.

Induction of noninsulin dependent diabetes mellitus

Noninsulin-dependent diabetes mellitus (NIDDM) was induced in overnight fasted adult Wistar albino male rats weighing 170–220 g by a single intraperitoneal injection of 60 mg/kg streptozotocin (STZ), 15 min after the i. p. administration of 120 mg/kg of nicotinamide. STZ was dissolved in citrate buffer with pH 4.5 and nicotinamide being dissolved in normal saline. Hyperglycemia in rats was confirmed by the elevated glucose levels in plasma, determined at 72 h and then on day 7 after injection. The starting value of fasting plasma glucose to identify diabetes was taken as >126 mg/dl. Only those rats which were found to have permanent NIDDM were used for the study.

Experimental

The animals were distributed into five different groups with six rats each. The extract was administered for 12 days. Group I served

as normal control rats were given drinking water daily for 12 days; Group II consisted of diabetic control rats administered drinking water daily for 12 days; Group III diabetic rats administered standard drug Glibenclamide (0.50 mg/kg) for 12 days; Group IV diabetic rats administered aqueous extract of 200 mg/kg body weight; and Group V diabetic rats administered aqueous extract of 400 mg/kg bodyweight. The fasting glucose levels were determined on day 0, 7, 14, and 28 of extract administration. During the experimental period, the rats were weighed daily, and the mean change in body weight was calculated.[17,18]

Estimation of biochemical parameters

On day 12, after the animals were sacrificed by cervical dislocation, the biochemical parameters were determined. The tests carried out were total cholesterol (TCL), triglycerides (TGL), high-density lipoprotein (HDL), low-density lipoprotein (LDL) by glucose oxidase method using autoanalyzer.[19,20]

Histopathology

All the animals were sacrificed on the 12th day by cervical dislocation, liver were isolated and were subjected to histopathological studies, and microscopical findings were noted.

Statistical analysis

The data are expressed as a mean ± standard error of the mean of six independent experiments. Statistical significance between group was evaluated using one-way analysis of variance followed by Dunnet's test and Duncan multiple range test with $P < 0.05$ value was considered as statistically significant.

RESULTS

Alpha-glucosidase inhibiting assay

The result of α-glucosidase inhibiting activity of aqueous extract of bark of FF is presented in Figure 1. Since the sample was extracted through four different methods to observe the variation in the bioactivity, it was found that the extraction process has a great influence on the bioactivity of plant extracts. The extract obtained through PB has showed the highest activity with the lowest IC_{50} values (10.25 ± 4.56 mg/ml FWT). While the CP extract exhibited the lowest activity.

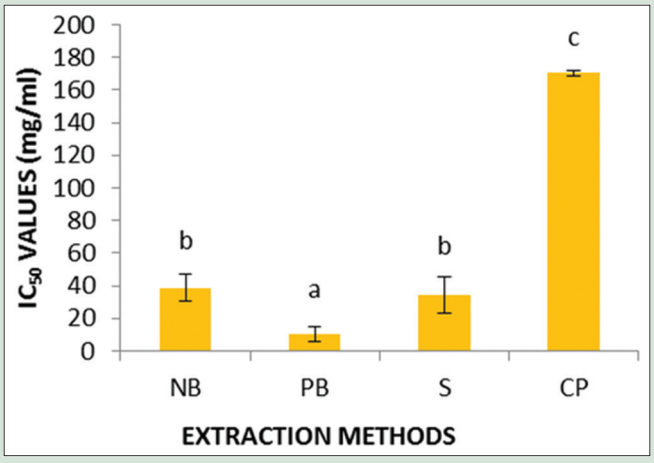


Figure 1: α-Glucosidase inhibiting activity of aqueous extracts of *Fraxinus floribunda*. Values with different letters (a, b, c) are significantly ($P < 0.05$) different from each other by Duncan multiple range test. NB: Normal boiling; PB: Pressure boiling; S: Soxhlet; CP: Cold percolation

Acute toxicity study

The acute toxicity study of aqueous FF bark extract was evaluated as per the CPCSEA guideline no. 420 (fixed dose method). The aqueous extracts were orally fed to the rats at the dose level of 5, 50, 300, and 1000 mg/kg, respectively. The test showed no mortality even at maximum dose of 1000 mg/kg body weight (b.w.). Hence, 200 mg/kg and 400 mg/kg doses were selected for further study.

Oral glucose tolerance test

Results obtained from OGTT is given in Table 1 where it can be observed that there is a significant increase of blood glucose concentration ($P < 0.05$) in the diabetic control group compared to the normal control at the end of 12th day experiment duration. The elevated glucose level was significantly lowered ($P < 0.05$) by the aqueous extracts of bark of FF while comparing with the diabetic control. The significant reduction in plasma glucose level in glucose-loaded rats by 200 and 400 mg/kg extract of FF bark was observed after 30 min, and it came down to normal level after 90 min.

Effect on fasting blood glucose

Table 2 shows administration of STZ-induced hyperglycemia in rats with statistically significant ($P < 0.05$) elevation of blood glucose as compared to normal control to the level >250 mg/dl. However, it was significantly ($P < 0.05$) lowered by the oral administration of glibenclamide (0.05 mg/kg) and aqueous extract of FF bark (200 and 400 mg/kg). As expected, 400 mg/kg of the extract showed the more significant antidiabetic property. The glucose level in STZ-induced diabetic rats lowered to normal after day 14 in case of glibenclamide and 400 mg/kg extract, whereas the 200 mg/kg extract could lower the glucose level to normal after day 28.

Body weight

Effect of standard drug and bark extract of FF on the body weight of diabetes-induced rats is shown in Table 3. In normal control rats, it was observed that bodyweight continuously increased. Diabetic control rats showed a significant decrease ($P < 0.05$) in bodyweight compared to normal control. The diabetic rats administered with glibenclamide and extract of FF bark (200 and 400 mg/kg) showed a significant increase ($P < 0.05$) in bodyweight when compared to diabetic control.

Effect of extracts on lipid profile

Lipid profile of the STZ-induced diabetic rats is presented in Table 4. It can be observed that TGL, TCL, and LDL level were significantly increased ($P < 0.05$) in diabetic rats as while HDL level was significantly decreased as compared to normal control. The bark extracts of FF (200 and 400 mg/kg) significantly decreased the serum TGL, TCL, and LDL and increased the HDL when compared with the diabetic control.

Table 1: Effect of different extracts on oral glucose tolerance test

Group	Plasma glucose concentration (mg/dl)		
	0 min	30 min	90 min
Normal control	73.15±3.24	76.42±2.73	74.83±4.51
Glucose control	76.61±6.17	221.28±5.38$^{\#}$	155.19±3.64$^{\#}$
Glucose + glibenclamide (0.5 mg/kg)	78.34±4.42	90.71±3.25*	75.52±5.18*
Extract (200 mg/kg)	75.58±3.71	128.21±4.86*	96.17±4.43*
Extract (400 mg/kg)	74.83±5.36	108.47±3.18*	78.63±3.29*

Values are expressed as mean±SEM (number of animals, n=6); Significantly different at $^{\#}P<0.05$ when compared with normal control group; $^{*}P<0.05$ when compared with diabetic control group. SEM: Standard error of the mean

Table 2: Effect of different extracts on fasting plasma glucose level in rats

Group	Fasting plasma glucose concentration (mg/dl)			
	Day 0	Day 7th	Day 14th	Day 28th
Normal control	78.62±2.15	75.39±5.12	80.17±3.41	74.69±5.28
Diabetic control (STZ)	149.29±3.62#	208.34±2.57#	249.48±5.62#	287.11±2.71#
Diabetic + standard glibenclamide (0.50 mg/kg)	134.43±2.83	112.65±4.32*	90.29±2.39*	75.38±4.68*
Diabetic + extract (200 mg/kg)	129.51±4.34	132.74±3.51*	113.68±4.73*	92.76±2.15*
Diabetic + extract (400 mg/kg)	132.24±3.05	118.43±4.26*	97.36±2.86*	78.42±3.52*

Values are expressed as mean±SEM (number of animals, *n*=6); Significantly different at #*P*<0.05 when compared with normal control group; **P*<0.05 when compared with diabetic control group. SEM: Standard error of the mean

Table 3: Effect of extracts on changes in bodyweight in rats

Group	Change in body weight (g)		
	Before induction	After induction	After treatment
Normal control	182.32±2.14	171.49±3.28	177.83±2.63
Diabetic control (STZ)	185.21±1.98	139.67±2.68#	112.38±3.86#
Diabetic + standard glibenclamide (0.50 mg/kg)	168.19±3.05	132.72±3.14	175.31±4.56*
Diabetic + extract (200 mg/kg)	176.15±3.53	142.59±2.43	151.84±2.49*
Diabetic + extract (400 mg/kg)	173.64±2.79	146.12±1.54	169.76±2.18*

Values are expressed as mean±SEM (number of animals, *n*=6); Significantly different at #*P*<0.05 when compared with normal control group; **P*<0.05 when compared with diabetic control group. SEM: Standard error of the mean; STZ: Streptozotocin

Table 4: Determination of biochemical parameters after treatment with different extracts

Group	Lipid profile (mg/dl)			
	TGL	TCL	HDL	LDL
Normal control	79.24±3.28	76.65±4.36	72.84±2.68	56.28±4.53
Diabetic control (STZ)	186.37±3.69#	198.41±2.72#	31.56±4.25#	165.69±5.74#
Diabetic + standard glibenclamide (0.50 mg/kg)	78.53±4.17*	72.84±4.69*	76.29±6.43*	63.82±2.28*
Diabetic + extract (200 mg/kg)	96.52±2.69*	101.62±2.81*	51.35±3.79*	85.26±4.15*
Diabetic + extract (400 mg/kg)	83.41±2.47*	79.95±5.24*	70.16±4.41*	58.64±3.26*

Values are expressed as mean±SEM (number of animals, *n*=6); Significantly different at #*P*<0.05 when compared with normal control group; **P*<0.05 when compared with diabetic control group. SEM: Standard error of the mean; TGL: Triglycerides; TCL: Total cholesterol; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; STZ: Streptozotocin

As expected, standard glibenclamide administered rats significantly prevented the increase of TGL, TCL, and LDL and decrease of HDL compared to diabetic control. The extracts were able to restore the lipid profile of diabetic rats to almost normal level.

Histopathology

The effect of bark extracts of FF on the histological architecture of the liver is given in Figure 2. Liver sections of the normal group showed normal hepatic structure [Figure 2a]. Normal hepatic cells were observed distinctively forming a network around central veins with peripheral portal areas in the surrounding. However, liver sections of diabetes-induced rats showed hepatocellular injury with the loss of normal architecture of the liver as compared to the normal group [Figure 2b]. Inflammation and vacuolization of cytoplasm were observed. There was dilation in the central vein along with dilation and congestion of blood sinusoids. There was also an enlargement of the space between the hepatocytes and sinusoidal dilation. This hepatic injury was observed to be almost recovered to normal by the extracts (200 and 400 mg/kg) of FF bark [Figure 2d and e]. The sinusoids were restored with the reduction of enlargement, inflammation of central veins were also reduced. Administration of glibenclamide on diabetic rats was able to repair the hepatic injury to almost like normal control [Figure 2c].

DISCUSSION

We have already discussed that aqueous decoction of the bark of FF is popularly used in traditional medicine for treating diabetic patients in some villages of Sikkim. Since there is no scientific validation to its usefulness, this work was designed to ascertain the scientific base for this. In the traditional system, the simple boiling method is used to extract the therapeutic activity of the plant, but this work has focused on the other methods of extraction to observe the influence of various extraction methods on the bioactivity of the plant. Phytochemicals present in plants such as flavonoids, glycosides, terpenoids, alkaloids, and carotenoids are often implicated as having antidiabetic effect.[21] However, extraction method plays a crucial role in the productivity of these bioactive phytochemicals since such health promoters must be obtained sufficiently with minimum damage from the raw material.[22]

The samples extracted through four different methods were subjected to α-glucosidase inhibiting assay. Alpha-glucosidase is an enzyme that plays a vital role in modulating PPHG by breaking down α-1,4-glucosidic linkages of disaccharides. The effects of α-glucosidase inhibitors and their use on delaying the generation of blood glucose after food uptake has been established by various authors.[23] A distinct variation in antidiabetic activity by the extracts was observed. The bark extract of FF obtained through PB showed the highest antidiabetic activity with IC_{50} value 10.25 mg/ml FWT. The range of IC_{50} value increased up to 170.27 mg/ml FWT exhibited by CP with lowest antidiabetic activity. Similar results were reported previously where boiling under high pressure has extracted the highest antioxidant activity in case of FF.[24] According to mass transfer phenomena and phase theories, pressure increases the permeability and solubility of plant tissue, and there is a diffusivity

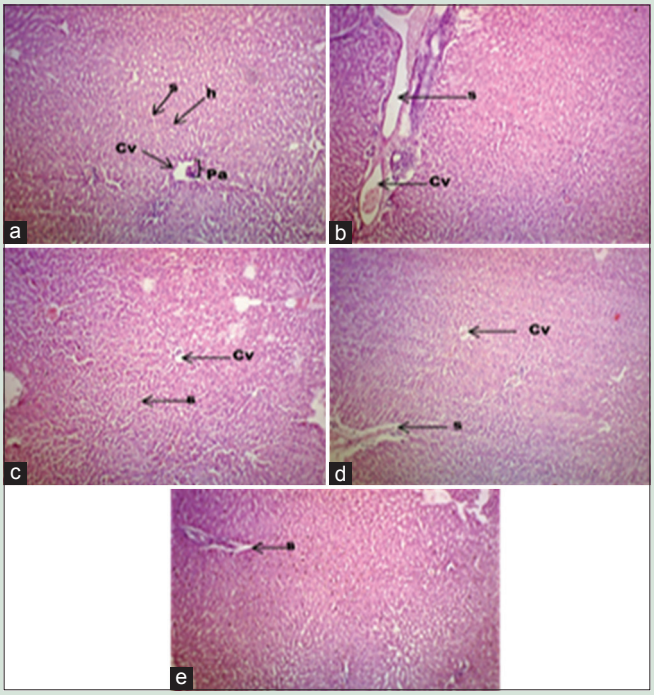


Figure 2: Liver histopathology of streptozotocin induced rats and effects of bark of *Fraxinus floribunda* on it. (a) Normal control, (b) Diabetic control, (c) Diabetic + glibenclamide, (d) Diabetic + 200 mg/kg extract, (e) 400 mg/kg extract. s: Sinusoids; h: Hepatocytes; Cv: Central vein; Pa: Portal area

of cell components which results in the movement of cellular components out of the cell.[25]

The extract obtained from PB was selected for further investigation of antidiabetic activity in animal model. There was an improvement in glucose tolerance test with the reduction of plasma glucose level which indicates the insulin-mimetic activity or improvement of glucose utilization mechanism by the extract.[26] The antidiabetic activity of FF extract might be credited to the decrease in damage of pancreatic β-cell, thus improving the production of insulin from the β-cell of the pancreas. Numerous plants have been previously reported to have antihyperglycemic activity by insulin stimulating effect.[27,28] The mechanisms of actions for plants with antihyperglycemic activity mainly include the increase of insulin secretion, control in glucose absorption by the intestine, more glucose absorption by muscle and fat tissues, and control in the production of glucose from liver cells.[29]

One of the characteristic features of diabetes is the inability of glucose uptake by the muscle cells because of low insulin production which consequently causes muscle wasting and decrease in bodyweight. Induction of STZ in rats will destroy the pancreatic β-cells due to low levels of insulin.[30] Thus, there was a decrease in bodyweight in diabetic control rats as compared to normal control ones, which indicate the excessive breakdown of tissue proteins causing the loss of body weight in diabetes.[31-34] It was clearly observed that the administration of FF extract improved the loss of bodyweight which indicated the control on wastage of muscle in diabetes. The FF extract has probably stimulated the pancreatic beta cells leading to the production of insulin.

Diabetes affects lipid profile, and the most common lipid abnormalities are high TGL and high TCL. In this study, there was an increase in TCL and decrease in HDL in diabetic control rats. Deficiency of insulin may cause the failure to activate lipoprotein lipase resulting into hypertriglyceridemia.[35] However, the bark extract of FF was able to control the lipid levels in diabetic rats. In diabetes, LDL brings cholesterol to the peripheral tissues to be deposited while HDL carries cholesterol to liver from peripheral tissues and helps its excretion. LDL is responsible for the deposition of fats in arteries. In this study, we have observed a significant decrease in TCL, TGL, and LDL, whereas HDL level was significantly increased.

The liver is important and helps the body in controlling blood glucose with glycogenesis and glycogenolysis. The liver sections of STZ-induced rats revealed various architectural changes in the liver with inflammation of sinusoids, changes in central veins, and portal area with vacuolization of cytoplasm. Similar findings were reported earlier by many researchers with histopathological changes in the liver.[36-38]

Unlike diabetic control, the liver sections of diabetic rats treated with glibenclamide and FF bark extracts showed less histopathological changes and improved liver architecture. It indicates the protective effect of the extracts to control hepatic injury during diabetes.

CONCLUSION

From the results of this study, it can be concluded that the aqueous extract of bark of FF possess antidiabetic activity along with antihyperlipidemic property against STZ-induced hyperglycemia. We also suggest extraction of the FF bark with a method involving pressure to acquire better results. Our study supports the traditional use of this plant for the treatment of diabetes.

Acknowledgement

We would like to thank UGC for providing financial support through Rajiv Gandhi National Fellowship. We would also like to express our gratitude toward Columbia Institute of Pharmacy, Raipur, Chhattisgarh, India for letting us perform the experiments on animal model in their laboratory. We would also like to acknowledge the Forests, Environment and W/L Management Department, Government of Sikkim, Gangtok for providing the permit for survey in West Sikkim. Most importantly, we are highly grateful to Mr. Tulshi Ram Sharma for enlightening us about the herbal use of *Fraxinus floribunda* and providing us the sample used in the experiment.

Financial support and sponsorship

Rajiv Gandhi National Fellowship, University Grants Commission.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Raman BV, Krishna AN, Rao BN, Saradhi MP, Rao MV. Plants with antidiabetic activities and their medicinal values. Int Res J Pharm 2012;3:11-5.
2. Elosta A, Ghous T, Ahmed N. Natural products as anti-glycation agents: Possible therapeutic potential for diabetic complications. Curr Diabetes Rev 2012;8:92-108.
3. Pavana P, Sethupathy S, Manoharan S. Antihyperglycemic and anti-lipid peroxidative effect of *Tephrosis purpura* seed extract in streptozotocin induced diabetic rats. Indian J Clin Biochem 2007;22:77-83.
4. Mlinar B, Marc J, Janez A, Pfeifer M. Molecular mechanisms of insulin resistance and associated diseases. Clin Chim Acta 2007;375:20-35.
5. Gupta PD, De A. Diabetes mellitus and its herbal treatment. Int J Res Pharm Biomed Sci 2012;3:706-21.
6. He ZX, Zhou ZW, Yang Y, Yang T, Pan SY, Qiu JX, *et al.* Overview of clinically approved oral antidiabetic agents for the treatment of type 2 diabetes mellitus. Clin Exp Pharmacol Physiol 2015;42:125-38.
7. Ahamad J, Naquvi KJ, Mir SR, Ali M, Shuaib M. Review on role of natural α-glucosidase inhibitors for management of diabetes mellitus. Int J Biomed Res 2011;2:374-80.

8. Onal S, Timur S, Okutucu B, Zihnioğlu F. Inhibition of alpha-glucosidase by aqueous extracts of some potent antidiabetic medicinal herbs. Prep Biochem Biotechnol 2005;35:29-36.
9. Li Y, Wen S, Kota BP, Peng G, Li GQ, Yamahara J, *et al. Punica granatum* flower extract, a potent alpha-glucosidase inhibitor, improves postprandial hyperglycemia in Zucker diabetic fatty rats. J Ethnopharmacol 2005;99:239-44.
10. Vijayaraj R, Vidhya R. Biological activity of *Achyranthes aspera* linn: A review. Asian J Biochem Pharm Res 2016;1:86-93.
11. Spiller HA, Sawyer TS. Toxicology of oral antidiabetic medications. Am J Health Syst Pharm 2006;63:929-38.
12. Eurich DT, McAlister FA, Blackburn DF, Majumdar SR, Tsuyuki RT, Varney J, *et al.* Benefits and harms of antidiabetic agents in patients with diabetes and heart failure: Systematic review. BMJ 2007;335:497.
13. Saxena A, Vikram NK. Role of selected Indian plants in management of type 2 diabetes: A review. J Altern Complement Med 2004;10:369-78.
14. Vijayaraj R, Kumaran NS. Evaluation of *in vivo* antidiabetic and antioxidant activity of *Achyranthes aspera* linn. seeds by streptozotocin induced diabetic rats. Int J Green Pharm 2018;12:29-37.
15. Subba A, Dutta B, Sahu RK, Mandal P. Antioxidant, anti-inflammatory and hepatoprotective activity of *Fraxinus floribunda* bark and the influence of extraction process on their bioactivity. J Pharm Res 2017;11:983-90.
16. Oki T, Matsui T, Osajima Y. Inhibitory effect of α-glucosidase inhibitors varies according to its origin. J Agric Food Chem 1999;47:550-3.
17. Singh J, Sahu RK, Prasad DN, Jangde R, Gupta R. Evaluation of antidiabetic potential of *Ougeinia oojeinensis* leaves in streptozotocin-induced-diabetic rats. Pharmacolonline 2011;2:1046-52.
18. Sharma U, Sahu R, Roy A, Golwala D. *In vivo* antidiabetic and antioxidant potential of *Stephania hernandifolia* in streptozotocin-induced-diabetic rats. J Young Pharm 2010;2:255-60.
19. Nyarko AK, Sittie AA, Addy ME. The basis for the antihyperglycaemic activity of *Indigo feraarrecta* in the rat. Phytother Res 1993;7:1-4.
20. Barham D, Trinder P. An improved colour reagent for the determination of blood glucose by the oxidase system. Analyst 1972;97:142-5.
21. Malviya N, Jain S, Malviya S. Antidiabetic potential of medicinal plants. Acta Pol Pharm 2010;67:113-8.
22. Kocak E, Pazır F. Effect of extraction methods on bioactive compounds of plant origin. Turk J Agric Food Sci Technol 2018;6:663-75.
23. Shobana S, Sreerama YN, Malleshi NG. Composition and enzyme inhibitory properties of finger millet (*Eleusine coracana* L.) seed coat phenolics: Mode of inhibition of alpha-glucosidase and pancreatic amylase. Food Chem 2009;115:1268-73.
24. Subba A, Mandal P. Antioxidant potential of *Fraxinus floribunda* bark extracted through various aqueous processing. Free Rad Antiox 2015;5:6-12.
25. Banerjee S, Chatterjee J. Efficient extraction strategies of tea (*Camellia sinensis*) biomolecules. J Food Sci Technol 2015;52:3158-68.
26. Singh MP, Shukla ST, Pateriya A. An investigation on antidiabetic and antioxidant activity of extract of leaves of *Amaranthus polygamus* in streptozotocin induced diabetic rats. Indian Drugs 2018;55:49-55.
27. Jia Q, Liu X, Wu X, Wang R, Hu X, Li Y, *et al.* Hypoglycemic activity of a polyphenolic oligomer-rich extract of *Cinnamomum parthenoxylon* bark in normal and streptozotocin-induced diabetic rats. Phytomedicine 2009;16:744-50.
28. Gireesh G, Thomas SK, Joseph B, Paulose CS. Antihyperglycemic and insulin secretory activity of *Costus pictus* leaf extract in streptozotocin induced diabetic rats and in *in vitro* pancreatic islet culture. J Ethnopharmacol 2009;123:470-4.
29. Girija K, Lakshman K, Udaya C, Sabhya SG, Divya T. Anti-diabetic and anti-cholesterolemic activity of methanol extracts of three species of *Amaranthus*. Asian Pac J Trop Biomed 2011;1:133-8.
30. Baruah D, Bora M, Bora D, Bharali BK, Hazra J. Evaluation of hypoglycemic activity of *Annona reticulata* L. stem bark extracts against streptozotocin induced diabetic rats. Int J Res Ayurveda Pharm 2017;8:132-6.
31. Sajeesh T, Arunachalam K, Parimelazhagan T. Antioxidant and antipyretic studies on *Pothos scandens* L. Asian Pac J Trop Med 2011;4:889-99.
32. Kumar D, Kumar S, Kohli S, Arya R, Gupta J. Antidiabetic activity of methanolic bark extract of *Albizia odoratissima* Benth. in alloxan induced diabetic albino mice. Asian Pac J Trop Med 2011;4:900-3.
33. Poongothai K, Ponmurugan P, Ahmed KS, Kumar BS, Sheriff SA. Antihyperglycemic and antioxidant effects of *Solanum xanthocarpum* leaves (field grown & *in vitro* raised) extracts on alloxan induced diabetic rats. Asian Pac J Trop Med 2011;4:778-85.
34. Mishra SB, Verma A, Mukerjee A, Vijayakumar M. Anti-hyperglycemic activity of leaves extract of *Hyptis suaveolens* L. poit in streptozotocin induced diabetic rats. Asian Pac J Trop Med 2011;4:689-93.
35. Appalaraju G, Reddy CS, Reddy CV, Rajani G, Nithin C, Raju AB. Anti diabetic activity of polyherbal extract on streptozotocin induced diabetes in Wistar rats. Ann Biol Res 2014;5:39-46.
36. Aboonabi A, Rahmat A, Othman F. Effect of pomegranate on histopathology of liver and kidney on generated oxidative stress diabetic induced rats. J Cytol Histol 2014;6:1-5.
37. Hassan NF, Soliman GM, Okasha EF, Shalaby AM. Histological, immunohistochemical, and biochemical study of experimentally induced fatty liver in adult male albino rat and the possible protective role of pomegranate. J Microsc Ultrastruct 2018;6:44-55.
38. Ania IM, Abireda AN, Mustafab BE, Wahaba EN, Azzubaidic MS. Effect of flaxseed extract on the liver histological structure in streptozotocin induced diabetic rats. Int Med J Malaysia 2017;6:91-8.