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Full Length Research Paper

Diversity among 20 accession of three germplasm of the medicinal plant, *Ocimum* (*O. gratissimum*, *O. sanctum* and *O. basilicum*, Lamiaceae)

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Ocimum spp, popularly known as Tulsi, has great commercial (food and perfumery industries) and medicinal importance due to antispasmodic, stomachic, carminative, antimalarial and febrifuge properties. The aroma and flavor in *Ocimum* spp. is distinct due to the predominant aroma compound eugenol, camphor, citral etc. We used molecular techniques to assess the genetic variability and relatedness of 20 accessions of three germplasm of *Ocimum* spp. (*Ocimum gratissimum*, *Ocimum sanctum* and *Ocimum basilicum*) collected from different places of India. DNA was isolated by fixing a sample in alcohol without using liquid nitrogen. 20 accessions were analyzed through random amplified polymorphic DNA (RAPD) profiling for similarity and genetic distance, using 18 primers. The binary (1/0) data was analyzed with REEPLIT to infer the genetic distance and to construct the unweighted pair group method with arithmetic mean (UPGMA) based dendrogram. High degree of polymorphism (82.78%) was shown by RAPD markers. There was total 122 bands generated, 101 bands were polymorphic. Highest similarity was measured at approximately 0.97% and least was 0.46%. The present work showed interesting finding and proved to be a bidirectional evolution in *Ocimum* species. Therefore, RAPD markers can be used in the systematic study of wild plants and new crop. The present study would provide suitable keys for further studies.

Key words: *Ocimum*, Tulsi, genetic diversity, degree of divergence, R marker.

INTRODUCTION

Tulsi (family: Lamiaceae, *Ocimum* spp.), the holy Indian basil, is widely distributed in tropical and subtropical regions of India. The plant contains multiple bioactive substances having several health promoting and disease preventing properties (Prakash and Gupta, 2005) that are attributed to the essential oils stored in peltate glands of leaves and stems. The essential oils comprise a number of aromatic chemicals like terpenoids and phenyl propanoids. These compounds individually and in combination impart aroma and fragrance, plant-insect and

plant-pathogen interactions and are used as antioxidants in pharmaceutical industries. The red colored Tulsi also contains anthocyanin that has application as food colourants. Tulsi is a complex, variable plant. Interspecific, intraspecific hybridization and polyploidy are very common for this plant resulting in diversity with great variations (Tilwari et al., 2013). Owing to a high degree of polymorphism exhibited by the species as also abundant cross pollination, a large number of species, subspecies, varieties and strains have come into existence, which

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Table 1. Germplasm accession of *Ocimum* species.

Species	Accession No.
<i>O. sanctum</i>	OS-1
	OS-2
	OS-3
	OS-4
	OS-5
	OS-6
	OS-7
	OS-8
	OS-9
<i>O. basilicum</i>	OS-10
	OS-11
	OS-12
	OS-13
	OS-14
	OS-15
<i>O. gratissimum</i>	OS-16
	OS-17
	OS-18
	OS-19
	OS-20

make the botanical nomenclature extremely difficult (Krishna, 1981).

In view of the great diversity, the various species have been classified in broad groups, based on the geographical sources, morphological and cytological features and chemical constituents. The plants get further complicated by the presence of chemical races that do not differ morphologically, but differ in aromatic chemical constituents. Considering its potential as an aromatic, medicinal and pigmented plant and lack of molecular information about this plant, the aim of this study is to evaluate the genetic variability within the species and to determine the extent of correlation between the random amplified polymorphic DNA (RAPD) profiles. Molecular genetic tools like bar coding, random amplified polymorphism DNA are reliable method for quality control of herbal material. Finger prints obtained by RAPD can be employed for identification of herbal drug at the molecular levels. In this study, genetic variability and RAPD techniques were used for the assessment of diversity for 20 different accessions with three germplasm collections assembled from different places in India, as a prelude to crop improvement

MATERIALS AND METHODS

Collection and maintenance of varieties

Representative set of 20 accessions of three species of *Ocimum*, group 1 of *Ocimum sanctum* with 10 accessions, group 2, *Ocimum basilicum* with 6 accessions and group 3 *Ocimum gratissimum* with

4 accessions from different location of Uttar Pradesh, India (Table 1), were used for assessment of diversity. The accessions were selected randomly, which were grown to flowering stages at University of Allahabad. Three seedlings of each accession were transplanted into pots in triplicates. Greenhouse plants were irrigated to pot capacity daily and maintained at day/night temperatures of 26 to 30°C and 18 to 21°C, respectively. Taxonomic identification of each accession was conducted by Botanical Survey of India, Allahabad.

Extraction of genomic DNA

Total genomic DNA was extracted by CTAB method with some modification (Doyal and Doyal, 1990). Fresh young leaves from nursery raised plant individual genotype/accession progeny were collected in ice box. One gram leaf tissue was fixed in alcohol before cetyl trimethylammonium bromide (CTAB) DNA extraction, making liquid nitrogen unnecessary (Sharma et al., 2003). The protocol was modified slightly from the standard one as *Ocimum* species have high amount of oil and secondary metabolites (Suman et al., 1999; Khanuja et al., 1999). Alcohol was allowed to evaporate followed by grinding the tissue with mortar and pestle and then transferred into 10 ml polypropylene centrifuge tube. It contained 3 ml pre warmed (65°C) DNA buffer (2% CTAB, 20 mM ethylenediaminetetraacetic acid (EDTA), 1.4 mM NaCl, 100 mM Tris HCl pH 8.0) with 0.6 volume of β -mercaptoethanol and 2% polyvinylpyrrolidone (PVP). The tubes were shaken and incubated at 65°C for 60 min. Equal volume of chloroform: Isoamyl alcohols (24:1, v/v) have been added and tubes were shaken end to end for 10 min to make emulsion, then centrifuged at 15,000 rpm for 10 min. Supernatant have been collected and subjected to RNase treatment. Then DNA was precipitated using 0.6 volume chilled isopropanol, tubes were shaken end to end until DNA fiber appeared, centrifuged at 8000 rpm and 4°C for 10 min. Pellets were washed with washing sol (70% ethanol + 10 mM ammonium acetate), dried and then dissolved into 1.0 ml Tris EDTA (TE) buffer. Ammonium acetate 7.5 mM (0.5 vol.) has been added and kept at -20°C for 30 min, centrifuged at 8,000 rpm, 4°C for 20 min and supernatants have been collected into new tubes. DNA was precipitated using 4.5 ml absolute ethanol and pellets were dissolved in 1.0 ml TE buffer. Quantification and purity measurements of DNA have been performed by using UV spectrophotometer (Ultrospec-4000) and also analyzing the DNA on 0.8% agarose gel alongside diluted uncut lambda DNA as standard.

DNA amplification

PCR amplification was carried out by using Master Cycler gradient Thermal Cycler (Eppendorf). Amplification was carried out in 50 μ l reaction volume containing 1 \times Taq polymerase buffer (finnzyme), 1.5 mM magnesium chloride 4.5 μ l, 200 M each dNTP (Finnzyme), 20 M primer, 1 unit of the Taq DNA polymerase enzyme (Finnzyme) and 50 ng of template DNA. Thermal cycler with an initial denaturation at 94°C for 3 min, followed by 42 cycles was done. Each cycle consisted of denaturation at 94°C for 45 s, primer annealing at 42°C for 1 min, extension at 72°C for 3 min, with final extension at 72°C for 8 min. PCR products were separated on 1.5% agarose gel in 1 \times TBE buffer using ethidium bromide staining. The size of amplified fragments was determined by using size standard (3 kb DNA ladder Finnzyme). DNA fragment were visualized under UV light and photographed using VSD Image master (Pharmacia Biotech). To test the reproducibility of the RAPD markers, the reactions were repeated in duplicate.

Statistical analysis

Amplicons were scored as discrete variables, using 1 for the

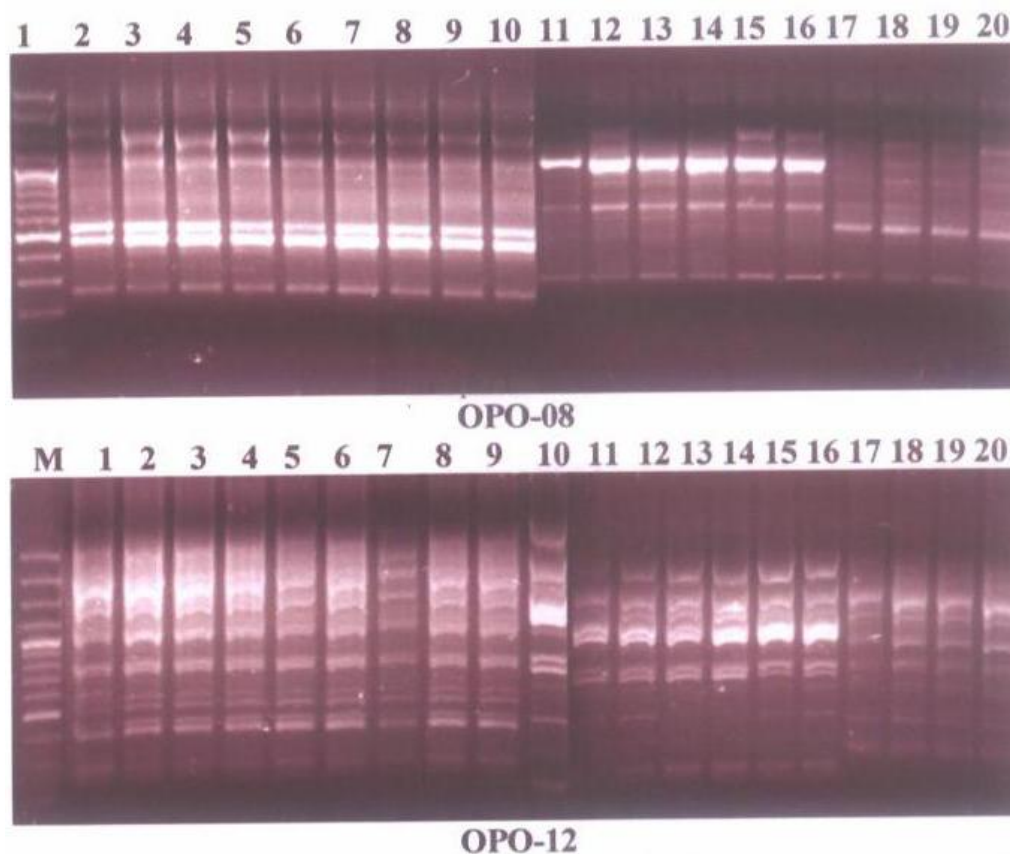


Figure 1. RAPD pattern of *Ocimum* spp.
Lane M: standard DNA marker (DNA); 1-20 represent accession from OS-1 to OS-20 by primer OPO-08 and OPO-12.

presence of bands and 0 for the absence of bands. Jaccard similarity coefficient based similarity matrix have been prepared by using the formula (Jaccard, 1908). The pattern of presence and absence of bands was submitted to NT-SYS-PC based software (SIMINT) to prepare similarity matrix. unweighted pair-group method of arithmetic average analysis (UPGMA) have been performed by SHAN which uses similarity matrix of SIMQUAL as input file. Phylogenetic tree based on similarity matrix have been viewed by using graphics (TREEPLOT).

RESULTS

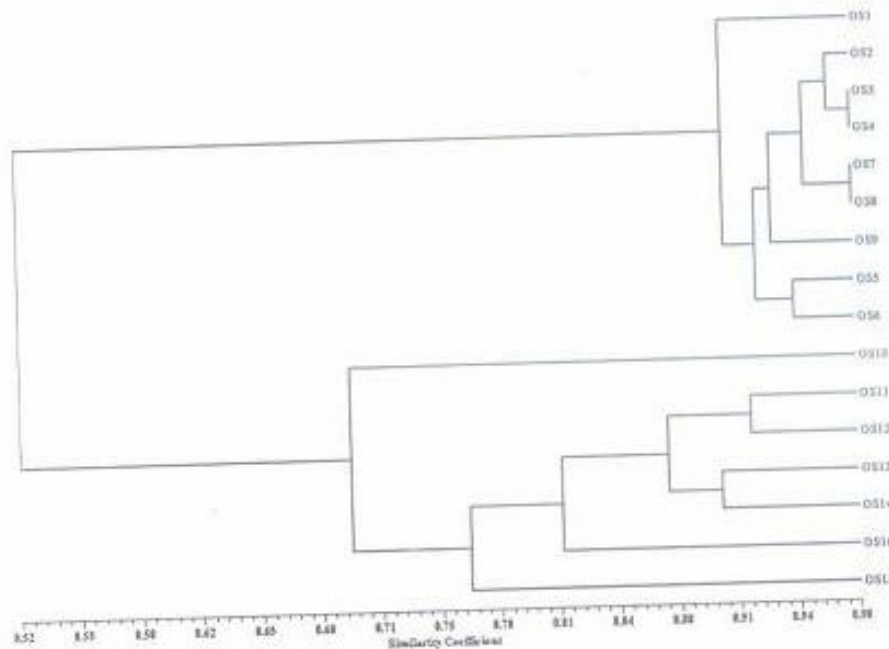
Each accession of three *Ocimum* species was considered as an individual operational taxonomic unit (OTU). Out of eighteen primers analyzed in the present study, fourteen random primers were found to produce scorable RAPD patterns and used for analysis. Two primers (OPO-17 and OPT-15) produced fuzzy and hardly repeatable bands while other two primers (OPT-10 and OPT-14) did not produce any amplification product and was thus excluded from the present study. A total 122 bands were scored from polymerase chain reaction (PCR) amplification of the genomic DNA of 20 accessions of three *Ocimum* species (Figure 1).

A high degree of polymorphism (82.78%) was shown by RAPD markers. It was observed that out of total 122 bands generated, 101 bands were polymorphic (Table 2). Average number of 9 bands were obtained per primer and amplification produced ranged in the size from 200 bp to 3.0 kb. Maximum number of 12 amplification product was obtained with the primer OPO-12, followed by 10 amplicons for Primers OPO-03 and OPT-12. Maximum number of RAPD products was obtained for Primer OPO-08 and OPT-06 (7 bands with each primer). 28 RAPD products were recorded as unique or species specific. An average of 82.78% polymorphism was shown by primers. However, 100% polymorphism was found with primers OPT-01 and OPT-06. The primer OPO-05 showed the least % of polymorphism in *Ocimum* accessions (62.50%). Though the primer OPO-08 showed a high degree of polymorphism (85.71%), its distinguishing capacity was found to be poor comparatively.

The result of RAPD analysis based genetic similarity matrix showed varying degree of genetic relatedness among *Ocimum* accessions belonging to three species. Highest similarity (0.97) was measured in the OS-4 and OS-3 and OS-7 and Os-8 accessions of *O. sanctum*. The

Table 2. Polymorphism shown by various primers.

S/No.	Primers	Oligo sequence	Total Number of Bands	Total number of polymorphic bands	Polymorphism (%)
1	OPO-02	5'ACGTAGCGT C 3'	8	6	75
2	OPO-03	5'CTG TTG CTA C 3'	10	9	90
3	OPO-04	5'AAG TCC GCT C 3'	9	8	88.8
4	OPO-05	5'CCC AGT CAC T 3'	8	5	62.50
5	OPO-08	5'CCT CCA GTG T 3'	7	6	85.71
6	OPO-12	5'CAG TGC TGT G 3'	12	8	66.6
7	OPO-13	5'GTC AGA GTC C 3'	9	6	66.6
8	OPO-14	5'AGC ATG GCT C 3'	7	6	85.71
9	OPO-18	5'CTC GCT ATC C 3'	9	8	88.88
10	OPO-01	5'GGG CCA CTC A 3'	9	9	100
11	OPO-06	5'CAA GGG CAG A 3'	7	7	100
12	OPO-12	5'GGG TGT GTA G 3'	10	9	90
13	OPO-13	5'AGG ACT GCC A 3'	8	6	75
14	OPO-20	5'GAC CAA TGC C 3'	9	8	88.88
		Total	122	101	82.78

**Figure 2.** Combined phenetic dendrogram prepared based on genetic similarities among *ocimum* species germplasm accession.

least similarity (0.46) was measured in OS-17 (accession *O. gratissimum*) and OS-10 (accession of *O. basilicum*) and OS-14 (accession of *O. basilicum*) and OS-9 (accession of *O. sanctum*). Multivariate (cluster) analysis of the genetic similarity data grouped accessions belonging to three *Ocimum* species into two major clusters (Figures 2 and 3). Cluster I (Figure 2) included all the accessions belonging to *O. gratissimum* and *O. sanctum*

(except OS-15 that is an accession of *O. sanctum* var. *tenuiflorum* and showed the closeness to second cluster). Cluster II (Figure 3) included all the accession belonging to *O. basilicum*. Maximum average genetic similarity between species was found inbetween *O. sanctum* var. *tenuiflorum* and *O. gratissimum*.

Intra-clustering between *O. sanctum* accessions and *O. basilicum* accessions showed two sub-clusters and OS-

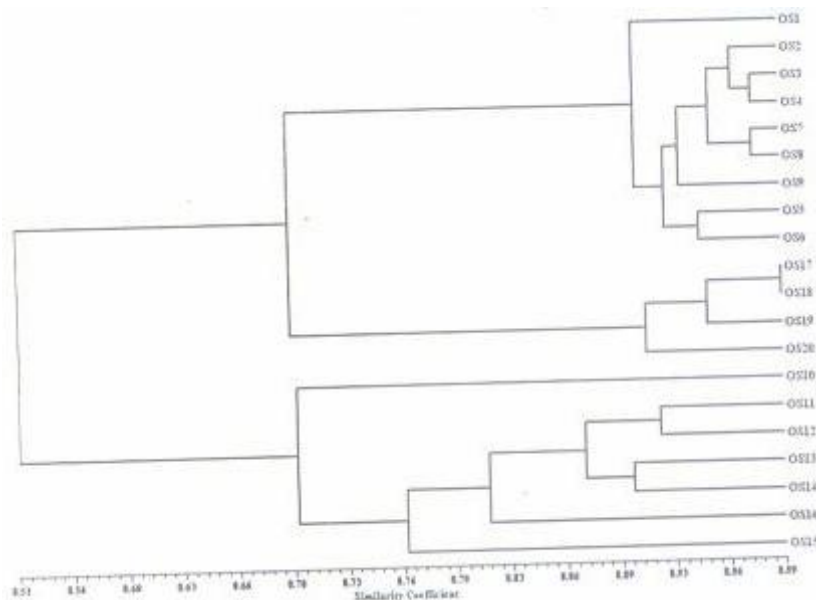


Figure 3. Interrelationship shown among the accessions *O. sanctum* and *O. basilicum*.

10 accession of *O. basilicum* showed closer genetic relatedness to *O. sanctum* cultivars (Figure 2). The combine dendrogram (Figure 3) showed nearly similar results obtained previously by other workers, who put the three *Ocimum* species into two main clusters and proving the closeness of *O. sanctum* accessions to *O. gratissimum* (Singh et al., 2004). Maximum primers used in the present study proved their capacity to distinguish different *Ocimum* species and their cultivars and hence, they are very useful in the assessment of biodiversity. It can give us intrageneric and intergeneric relationships among different species.

DISCUSSION

RAPD markers proved their advantage over morphological and chemical/biochemical (*isozyme*) markers because they detect maximum number of genetic loci (Sinde et al., 2007; Kongkiatngam et al., 1995). This is why they are applicable widely in various studies related to biodiversity assessment in laboratory. Another positive factor of RAPD is that it is less time and labor consuming, less expensive and can be easily perform (Khanuja et al., 1998). However some doubts have also been raised regarding the suitability of RAPD for diversity analysis. It is debated that most of the co-migrating RAPD bands may not be allelic or composed of similar sequences (Bowbitch et al., 1993). On the other hand, a study in some species of *Glycine max* and *Allium* have demonstrated the homology of co-migrating RAPD band (William et al., 1993). Although, investigation on genetic diversity,

interrelationship and phylogeny of *Ocimum* have been reported earlier (Khosla, 1995; Viera et al., 2001; Tilwari et al., 2013). They have also analyzed the genetic diversity of *O. gratissimum* L. at DNA level using RAPD marker. The present study also analyzes the genetic diversity at molecular level using RAPD assay. RAPD marker have been shown to detect high polymorphism then restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) marker (Vid et al., 2007; Sang et al., 2009; Thormann et al., 1994; Das et al., 1999).

In the present study, RAPD marker revealed high degree of polymorphism (82.78%) among the 20 accession belonging to 3 *Ocimum* species. it may be due to the possibility that most of the genetic loci is screened with RAPD markers were tone respected or of more species. *Ocimum* species were grouped in the present paper into two major clusters, one belonging to the accession of *O. basilicum*.

Previously, on the basis of morphological, cytological and oil characters, other authors have divided *Ocimum* species into two groups- '*Basilicum*' and '*Sanctum*'. *O. basilicum* and other species were included in the '*Basilicum*' group, while *O. tenuiflorum* and *O. gratissimum* were placed in the '*Sanctum*' group. The two clusters defined in the present study on the basis of similarity matrix obtained through RAPD analysis also corresponded to previously reported '*Basilicum*' and '*Sanctum*' groups, respectively. Hence, genetic divergence estimation among three *Ocimum* species under investigation suggests that phylogenetically, *O. basilicum* accessions are distantly related to other two species *O.*

O. Sanctum var. tenuiflorum and *O. gratissimum*. The result is indicative of a probably bidirectional evolutionary concept in *Ocimum* species. The study also proved that DNA can be easily extracted from plants by fixing leaves in absolute alcohol without impairing its quality for routine molecular biological work and without requirement of liquid nitrogen for DNA isolation. The study may be a preliminary step in understanding the vast area of biodiversity assessment in plant science.

Conclusion

Biodiversity assessment of *Ocimum* species on various morphological and molecular marker systems is of interest to researchers since a long time, because of its rich medicinal properties. RAPD molecular marker has been taking advantage over other techniques because it requires no prior knowledge about any particular gene in a target taxon. The present work showed interesting finding and proved a bidirectional evolution in *Ocimum* species. Therefore, RAPD markers can be used in the systematic study of wild plants and new crop and the present study would provide suitable keys for further studies.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Antifungal metabolite from *Muntingia calabura* root against early leaf blight of tomato

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The aim of the present study is to determine the *in vitro* antimicrobial activity of various extracts and fractions of *Muntingia calabura* (Elaeocarpaceae) root against a selected panel of microorganisms. Antifungal activity of different solvent extracts of *M. calabura* L. root, tested against *Alternaria solani*, *Fusarium oxysporum* f.sp. *lycopersici*, *Pythium* species, *Phytophthora* species, *Aspergillus niger*, *Colletotrichum* species and *Rhizoctonia solani*, was evaluated by agar well diffusion assay. The chromatographic fractionation of the extract resulted in the isolation of antifungal metabolite stigmasterol. The structure of the stigmasterol was confirmed using GC-MS, IR and NMR spectroscopic characterization. The stigmasterol had a potent antifungal activity with a minimum inhibitory concentration of 1 mg/ml against *A. solani*. Stigmasterol was subjected to docking studies carried out against fungal elicitor cryptogein. A better docking score of 12.59 with glide energy -42.56 was obtained for the complex fungal elicitor cryptogein. The interaction was done in chain A Tyr 47 [O – H...O] residue with a distance of 2.7 Å.

Key words: *Muntingia calabura*, minimum inhibitory concentration, phytopathogens, cryptogein elicitor, stigmasterol.

INTRODUCTION

Plant being a major source of natural therapeutic remedies, has been used in various part of the world to treat infectious diseases (Vahidi et al., 2002). Recent focus of research for new source of safer and more effective antibacterial agents has been shifted towards natural products of plant sources (Souza et al., 2002; Nitta et al., 2002). Higher plants, which are able to produce photosynthesis, produce hundreds to thousands of diverse chemical compounds with different biological activities (Hamburger and Hostettmann, 1991). It is believed that these compounds have an important ecological role. These antimicrobial compounds produced by plants are

active against plant and human pathogenic microorganisms (Sarac and Ugur, 2007). There are several reports in the literature regarding the antimicrobial activity of plant crude extracts and the bioassay-guided fractionation of those extracts that yielded active principles (Rabe and Van Staden, 2000; Palombo and Semple, 2001; Portillo et al., 2001; Srinivasan et al., 2001; El-Seedi et al., 2002; Zgoda-Pols et al., 2002).

Muntingia calabura L. (Kerukup siam), also known locally as Jamaica cherry, is a plant of the family Elaeocarpaceae (Morton, 1987). It is native to the American continent and is widely cultivated in warm

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areas of Asian region. The leaves, barks and flowers are believed to possess medicinal value as reported in Peru folklore medicinal uses. The roots have been employed as an emmenagogue in Vietnam and as an abortifacient in Malaysia (Chin, 1989). Various parts of this tree have several documented medicinal uses in both Southeast Asia and tropical America (Nshimo et al., 1993). The roots of *M. calabura* L. (Elaeocarpaceae) were investigated as part of a continuing project to discover novel antineoplastic agents of plant origin (Kaneda et al., 1991). From a cytotoxic Et₂O-soluble extract of *M. calabura* roots, twelve new flavonoids were isolated, constituting seven flavans, three flavones and two biflavans. Scientifically, several types of flavonoids and flavones have been isolated and identified from this plant (Su et al., 2003; Chen et al., 2005). Therefore, the main objective of this study is to search for the active fraction with strong antimicrobial activity which could serve as a good candidate for the development of new antimicrobial agents.

MATERIALS AND METHODS

Plant

The plant specimen with the root of *M. calabura* was identified and certified by the Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. Voucher specimens were maintained for future reference.

Microorganisms

The selected fungal pathogens including *Alternaria solani*, *Fusarium oxysporum* f.sp *lycopersici*, *Pythium* species, *Phytophthora* species, *Rhizoctonia solani*, *Aspergillus niger* and *Colletotrichum* species used in the present study was obtained from Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore.

Extract preparation

The dried and powdered root samples of *M. calabura* L. were extracted by overnight percolation with methanol (polar solvent), chloroform (medium polar solvent) and petroleum ether (least polar solvent) at the rate of 1:5 at room temperature. The extracts were then filtered and concentrated under vacuum in a rotary evaporator to obtain a gummy residue.

Antifungal studies by agar well diffusion assay

The antifungal activity of prepared extracts was tested against selected pathogens on potato dextrose agar. The fungal broth culture 200 µl was transferred to the Petri plates. Using sterile cork borer, wells of 6 mm in diameter were made in the plate containing the media. For each organism, 20 µl of the prepared sample dissolved in ethanol was loaded in each well using sterilized dropping pipette. Three replications were maintained for each treatment. For each microorganism, the positive control (ketoconazole) and the negative control (ethanol) were also loaded in a separate well. The plates were incubated and observed for 2 to 3 days. The diameter of inhibition zone (DIZ) was measured and the mean DIZ was calculated (Ameer et al., 2007; Iqbal and Faiz, 1998).

Fractionation and antifungal assay

The fractionation was carried out by column chromatography using a 22 cm long column having 1.6 cm internal diameter packed with 20 g of silica gel (60 to 120 mesh) using petroleum ether solvent. The active methanol extract was loaded to the packed column and elute with a mixture of petroleum ether and ethyl acetate mixture (90:10) at a rate of 2.6 ml min⁻¹. The polarity of mobile phase was gradually increased using ethyl acetate to get different fractions. Fractions with similar thin layer chromatogram were pooled together which were labeled serially and then subjected to antifungal assay to obtain the active fraction.

Minimum inhibitory concentration (MIC) by tube dilution method

Tube dilution method was used to obtain the MIC of methanol extract of *M. calabura* against selected fungal pathogen (Claeys et al., 1988). The MIC is defined as the lowest concentration of antibiotics or plant extracts that did not show any growth of tested pathogens. The entire test sample were dissolved in ethanol to dilute the highest concentration (10 mg/ml) to be tested and then serial dilutions were made to get 4 different concentrations of 10, 1, 0.1 and 0.01 mg/ml in sterile test tubes containing standardized inoculums. The growth of the organism for each dilution was observed and thus the MIC was evaluated. Ketoconazole and ethanol were used as positive and negative control, respectively.

Spectroscopic studies

The active fraction obtain from the methanol extract was subjected to gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) and infrared spectrometry (IR) spectroscopic studies to identify the structure of the active compound. GC-MS analysis was carried out by using Perkin Elmer - Clarus 500 GC-MS unit. The column used was TR 5-MS Capillary Standard Non-Polar Column with a dimension of 30 Mts, ID of 0.25 mm, film of 0.25 µm and helium as a carrier gas. The flow maintained was 1.0 ml min⁻¹ with oven temperature 80°C raised to 280°C at the rate of 10°C min⁻¹. The volume of sample injected was 2 µl. ¹H-NMR and ¹³C-NMR spectrums were recorded at 400 and 100 MHz, respectively in CDCl₃. The NMR spectroscopic studies were done by Bruker 400 spectrometer. The Fourier transform infrared (FT-IR) measurement of active fraction was performed using the Nicolet Avatar Model FT-IR spectrophotometer in a diffuse reflectance mode at a resolution of 4 cm⁻¹ in KBr pellets.

Molecular modeling studies

Induced Fit Docking (IFD) studies have been carried out using GLIDE (Jitendra and Vinay, 2011) software v5.5, developed by Schrodinger, running on Red Hat Enterprise Linux 5 (RHEL5) workstation and Maestro v9.0 Graphical User Interface (GUI) workspace was used for all the steps involved in ligand preparation, protein preparation and IFD.

Preparation of the ligand "fungal elicitor cryptogein"

The ligand used in this study was prepared using Ligprep module of v2.3 of Schrodinger Suite 2009. Ligprep follows OPLS-AA (Optimized Potential Liquid Simulations for All Atoms) force fields for energy minimization. The protein taken for the study was 1LRI (Engineered beta cryptogein complexed with cholesterol) retrieved from PDB database. The optimized structure was then energy

Table 1. Antimicrobial activity of *Muntingia calabura* root extract against fungal plant pathogens.

Extract	Zone of inhibition (Diameter in cm)						
	<i>Alternaria solani</i>	<i>F.oxysporum</i> f.sp. <i>lycopersici</i>	<i>Pythium</i> sp.	<i>Phytophthora</i> sp.	<i>Rhizoctonia solani</i>	<i>Aspergillus niger</i>	<i>Colletotrichum</i> sp.
Methanol extract (100 mg/ml)	2.3 (± 0.17)	2.0 (± 0.46)	2.0 (± 0.41)	1.8 (± 0.12)	1.5 (± 0.17)	1.6 (± 0.09)	1.7 (± 0.06)
Chloroform extract (100 mg/ml)	1.5 (± 0.12)	1.4 (± 0.06)	1.5 (± 0.07)	1.2 (± 0.12)	1.0 (± 0.06)	1.2 (± 0.17)	1.2 (± 0.23)
Petroleum ether extract (100 mg/ml)	1.0 (± 0.12)	0.7 (± 0.06)	0.8 (± 0.12)	0.6 (± 0.17)	0.6 (± 0.18)	0.7 (± 0.06)	0.6 (± 0.12)
Ketoconazole (1 mg/ml)	3.5 (± 0.64)	3.0 (± 0.29)	2.8 (± 0.55)	3.0 (± 0.29)	3.2 (± 0.64)	3.5 (± 0.52)	2.9 (± 0.58)
Ethanol (Control)	0.3 (± 0.12)	0.3 (± 0.07)	0.4 (± 0.03)	0.3 (± 0.06)	0.4 (± 0.09)	0.3 (± 0.12)	0.4 (± 0.02)

Values are Mean ± SD of three replications

minimized to remove the steric clashes between the atoms. The energy minimization was done till it reached a Root Mean Square Deviation (RMSD) cutoff of 0.18 Å and the resulting structure was used for docking (Thangaraj, 2011).

Induced fit docking (IFD)

IFD of the prepared ligand with the prepared protein was performed using IFD protocol of GLIDE v5.5 from Schrodinger Suite 2009. Both the ligand and the receptor were flexible which enabled the ligand to dock at the receptor's binding site and generate multiple poses of the receptor-ligand complex. Each docking included unique structural conformations of the receptor needed to fit the ligand pose. The IFD gives the best structure of the docked complex based on the Glide score (G-score) of the dockings.

RESULTS

The root of *M. calabura* L. possesses several secondary metabolites with remarkable biological properties. With this background the present research has been initiated to identify the active secondary metabolite compound produced by *M. calabura* L. root against selected plant pathogens.

Antifungal activity and fractionation

The various extracts of *M. calabura* root were

screened for their antifungal activity against selected plant pathogens. Among the three extracts tested, methanol extract exhibited the highest zone of inhibition when compared with chloroform and petroleum ether extract at a concentration of 100 mg/ml against the selected pathogens. The diameter of inhibition zone produced by the methanol extract (Table 1) was comparable to that of the positive control ketoconazole. The crude extract obtained from methanol extract of *M. calabura* roots were subjected to column chromatography for purification purpose. The polarity of petroleum ether was gradually increased using ethyl acetate up to 82:18 (Petroleum ether:Ethyl acetate) to give 40 different fractions. Fractions with similar TLC chromatogram were pooled together and as a result a total of 23 different fractions were obtained which were labeled as F1-F23. The collected fractions were tested for their antifungal activity against selected plant pathogens and the findings revealed that fraction No. 21 exhibited maximum antifungal activity when compared with other fractions (Table 2).

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration assay was

carried out for isolated antifungal fraction F21 against selected plant pathogens. The bioactive fraction F21 showed inhibition at a concentration of 10 and 1 mg/ml against the pathogens *A. solani*, *F. oxysporum* and *Phytophthora* spp., while growth was observed in the other two dilutions 0.1 and 0.01 mg/ml (Table 3).

Spectroscopic studies

The antifungal compound isolated from the root of *M. calabura* L. was obtained as whitish slight yellow colored compound. The spectroscopic studies confirmed the isolated active metabolite as stigmaterol. The molecular formula was determined as C₂₉H₄₈O by GC-MS studies (Figure 1) with m/z value of 412. The fragmentation pattern confirmed the presence of stigmaterol. The IR absorption spectrum showed absorption peaks at 3470 cm⁻¹ (O-H stretching), 2867.9 cm⁻¹ (aliphatic C-H stretching), and 1704.6 cm⁻¹ (C=C absorption peak). These absorption frequencies resemble the absorption frequencies observed for stigmaterol (Figure 2). In the ¹H-NMR spectrum, the six methyl groups appeared as, two singlet of three proton integration at δ 0.68 and 0.98 and were assigned to H-18 and H-19 of the tertiary methyl groups.

Table 2. Antifungal activity of the fraction separated from ethanolic extract of *M. calabura* root extract by column chromatography against fungal pathogens by agar well diffusion assay.

Fraction No.	Diameter of inhibition zone in cm					
	<i>A. solani</i>	<i>F. oxysporum</i> f.sp. <i>lycopersici</i>	<i>Pythium</i> sp.	<i>Phytophthora</i> sp.	<i>A. niger</i>	<i>R. solani</i>
F1	-	-	-	-	-	-
F2	-	-	-	-	-	-
F3	-	-	-	-	-	-
F4	-	-	-	-	-	-
F5	-	-	-	-	-	-
F6	-	-	-	-	-	-
F7	-	-	-	-	-	-
F8	-	-	-	-	-	-
F9	-	-	-	-	-	-
F10	-	-	-	-	-	-
F11	-	-	-	-	-	-
F12	-	-	-	-	-	-
F13	-	-	-	-	-	-
F14	0.2	0.2	0.2	0.2	0.2	0.2
F15	0.3	0.2	0.3	0.2	0.2	0.2
F16	-	-	-	-	-	-
F17	-	-	-	-	-	-
F18	-	-	-	-	-	-
F19	-	-	-	-	-	-
F20	0.4	0.3	0.3	0.3	0.3	0.3
F21	3.2	3.0	2.5	2.1	1.9	1.7
F22	0.3	0.2	0.3	0.3	0.3	0.3
F23	-	-	-	-	-	-
Ethanol (Control)	0.3	0.4	0.3	0.4	0.3	0.3
Ketoconazole (1 mg/ml)	3.5	3.1	2.8	3.1	2.4	3.2

Three secondary methyl groups resonated as doublets at δ 1.00 (J=6.5 Hz), δ 0.82 (J=6.0 Hz) and δ 0.77 (J=6.0Hz), and were assigned to H-21, H-26, H-27, respectively, whereas one primary methyl group appeared as triplet at δ 0.78 (J=7.5 Hz) and was ascribed to the H-29 of the side

chain. The appearance of a one proton multiplet at a downfield value of δ 3.51 in the $^1\text{H-NMR}$ spectrum revealed the presence of tertiary proton attached to hydroxyl group (Figure 3). Moreover, the $^{13}\text{C-NMR}$ indicated 29 carbon signals. The corresponding carbon signal, that is, C-3 was

indicated at δ 71.8 in the $^{13}\text{C-NMR}$ spectrum. The presence of a steroid skeleton was further confirmed by the $^{13}\text{C-NMR}$ signals at δ 130.2 and 138.3 and δ 140.7 and 121.7. $^{13}\text{CNMR}$ (Figure 4) (CDCl_3 , 100MHz): $^{13}\text{C-NMR}$ has given signal at 139.8 (C-5), 137.6 (C-22), 118.3, (C-6), 71.1 (C-3),

Table 3. Minimum inhibitory concentration of stigmasterol isolated from *M. calabura* root extract against fungal plant pathogens.

Extract	Fungal plant pathogens						
	<i>Alternaria solani</i>	<i>F.oxysporum</i> f.sp. <i>lycopersici</i>	<i>Pythium</i> sp.	<i>Phytophthora</i> sp.	<i>Rhizoctonia solani</i>	<i>Aspergillus niger</i>	<i>Colletotrichum</i> sp
Stigmasterol (mg/ml)							
10	-	-	-	-	+	+	+
1	-	-	+	-	+	+	+
0.1	+	+	+	+	+	+	+
0.01	+	+	+	+	+	+	+
Ketoconazole (mg/ml)							
10	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-
0.1	+	+	+	+	+	+	+
0.01	+	+	+	+	+	+	+
Solvent control	+	+	+	+	+	+	+
Cells	+	+	+	+	+	+	+

+ Growth; - No growth.

55.3(C-14), 55.18(C-17), 50.45 (C-9), 48.3 (C-9), 40.8 (C-20), 40.1(C-12),39.2 (C-13), 38.9 (C-4), 38.6 (C-12), 37.18 (C-1), 37.12 (C-10), 36.3(C-8), 35.59(C-20), 34.29 (C-22), 34.24 (C-7), 32.66 (C-8), 29.86 (C-25), 29.71 (C-16), 28.2 (C-2), 28.1 (C-15), 27.4 (C-28), 26.1 (C-11,26), 21.6 (C-27), 19.32 (C-19), 17.71 (C-21), 15.6 (C-18, 29), 130.2 (C-23). All the spectral data and previous literature reveal that the compound is stigmasterol (Figure 5).

Docking studies

IFD between the target protein beta-cryptogein (PDB ID-1LRI) and the compound stigmasterol was carried out using glide and the images are obtained using Pymol. This study clearly indicates that stigmasterol can be easily bind with the

cryptogein elicitors secreted by the pathogens and can prevent plant from pathogen attack. The possible conformations of compound stigmasterol along with their docking score and glide energy are shown in Table 4. The stigmasterol binds with cryptogein elicitors with a docking score value of -12.25 and glide energy of -42.56. The interaction was done in chain A Tyr 47 [O – H...O] residue with a distance of 2.7 Å° (Figure 6).

DISCUSSION

The root of *M. calabura* L. is a commonly used botanical medicine. The present study has explored the use of *M. calabura* in the field of agriculture, by using it against selected plant pathogens. A new antifungal compound, stigmasterol was identified with a minimum inhibitory

concentration of 1 mg/ml against the selected pathogens, namely, *A. solani*, *F. oxysporum* f.sp. *lycopersici*, and *Phytophthora* spp.

There is growing evidence that most of the secondary metabolites from plants are involved in the defense of the plant from plant pests and diseases. Thus, secondary compounds represent a large reservoir of chemical structures with biological activity. This resource is largely untapped for use as pesticides and fungicides. Many of the medicinal plants have been found to possess antimicrobial activity against an array of plant pathogens (Okigbo and Nmeke, 2005; Siva et al., 2008). Some plants may be alternatives to currently used disease control agents, since they constitute a rich source of bioactive chemicals (Swain, 1977). The substances, which can either inhibit the growth of pathogens or kill them and have no or least toxicity to host cells are considered as candidates

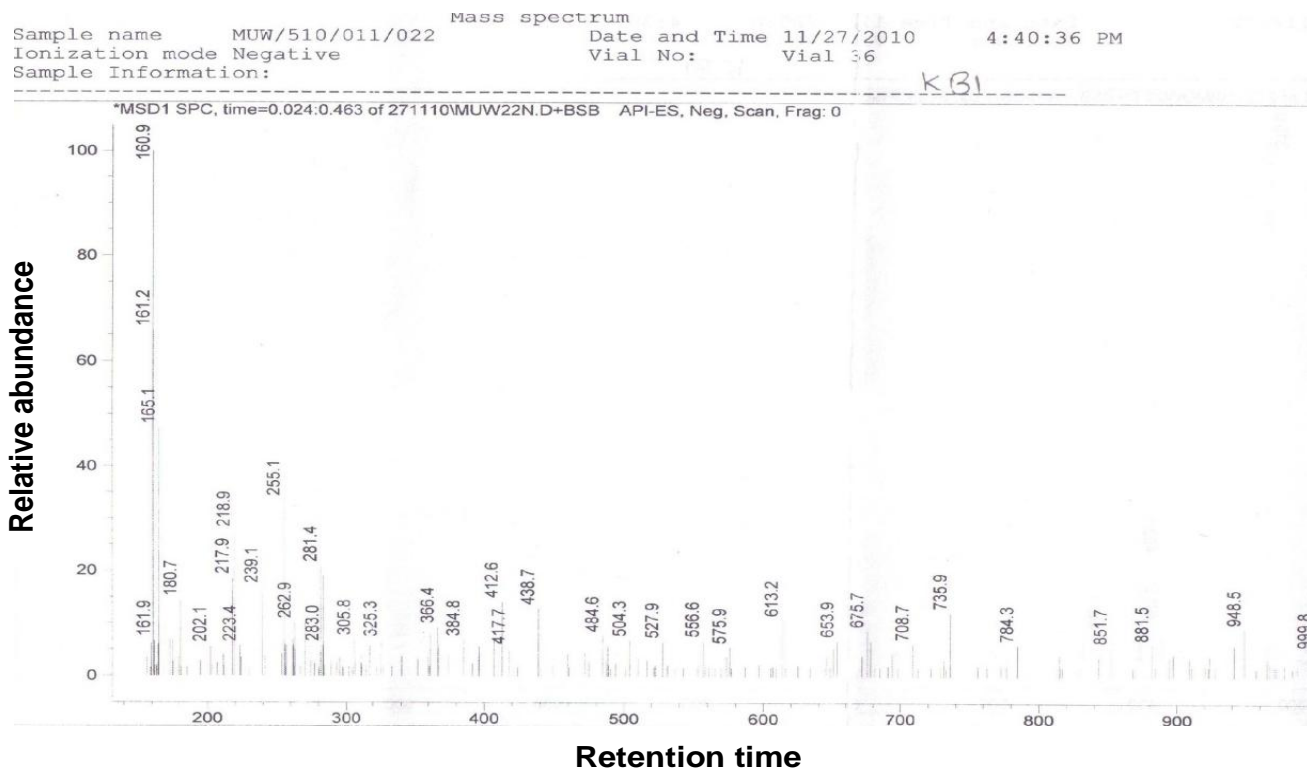


Figure 1. Gas chromatographic mass spectrum of purified antimicrobial fraction F21 from *M. calabura* root extract.

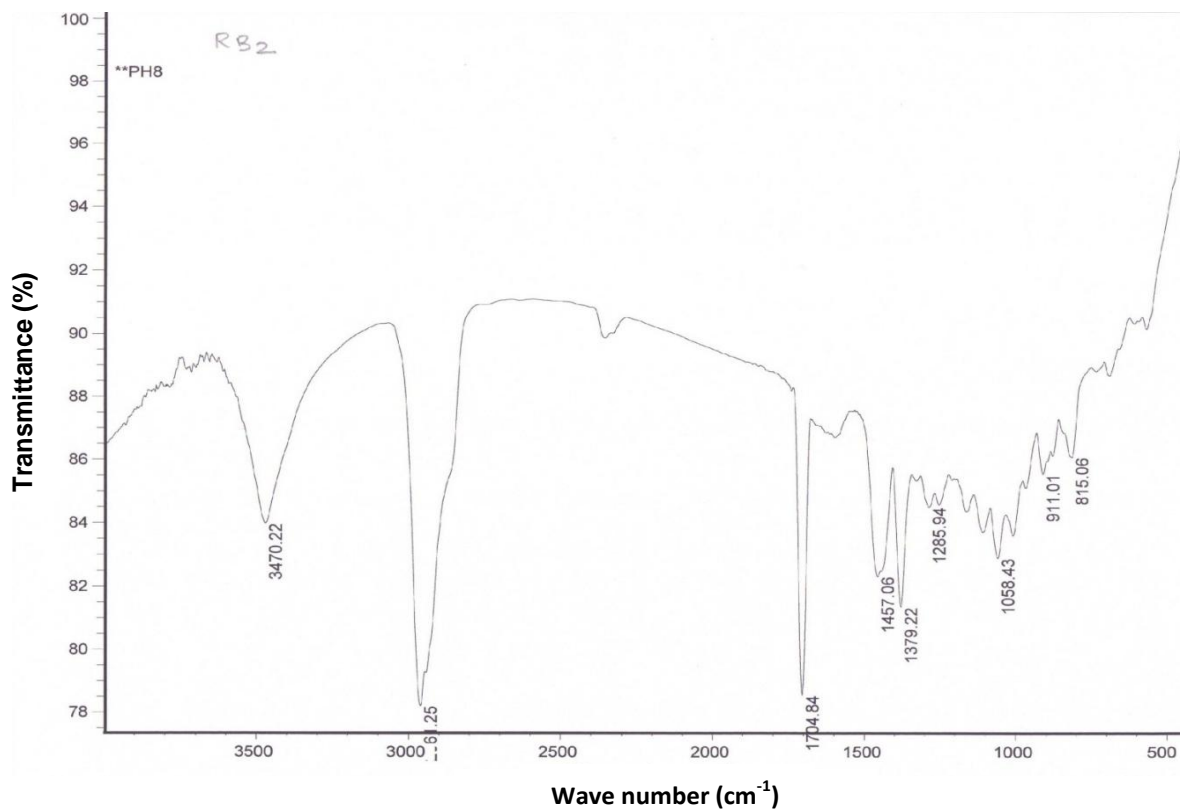


Figure 2. Infrared (IR) spectrum of purified antimicrobial compound from *M. calabura* root extract.

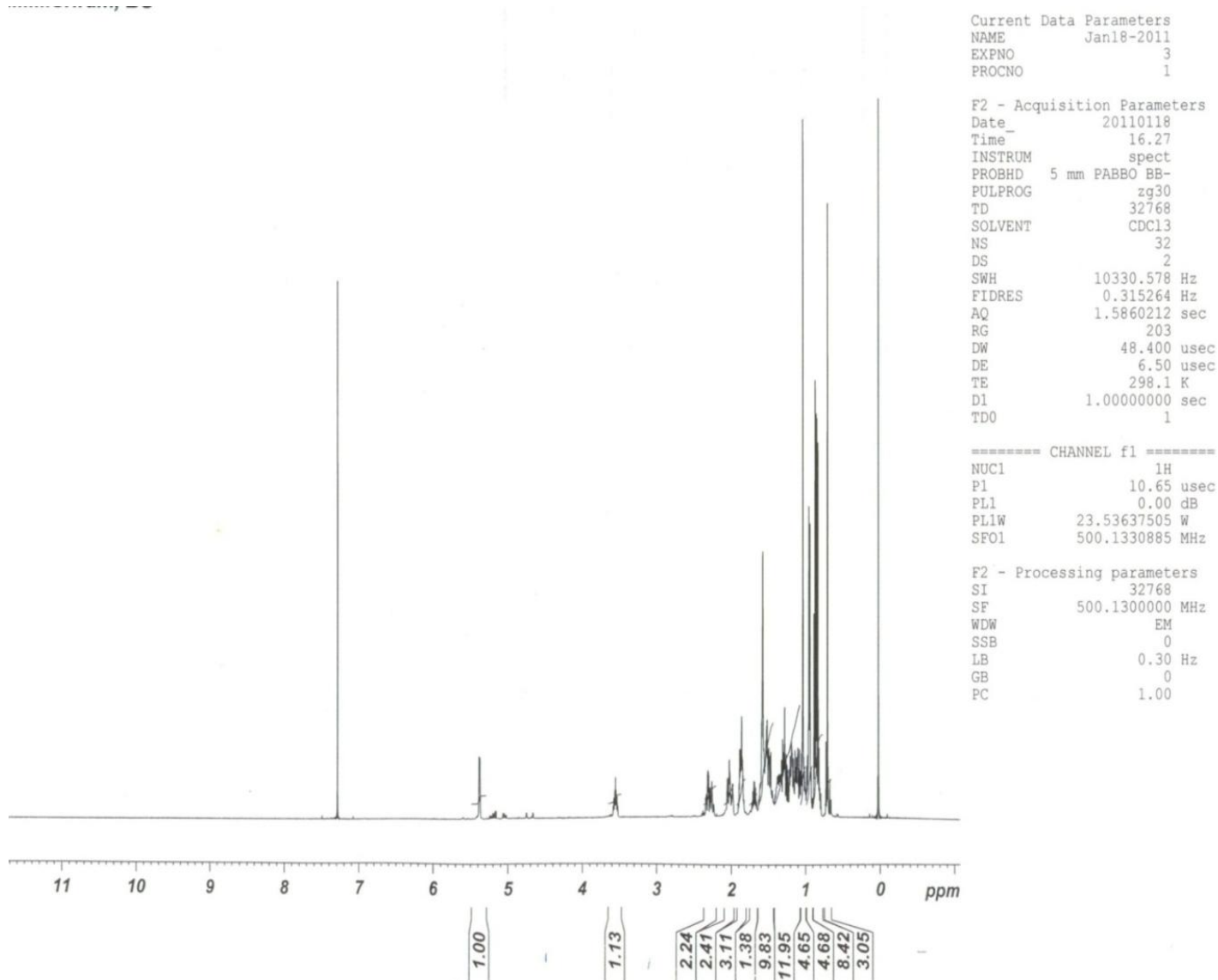


Figure 3. ^1H NMR studies of purified antimicrobial fraction F21 from *M. calabura* root extract.

Determination of antimicrobial activity of *M. calabura* root was tested against selected fungal plant pathogens by agar well diffusion assay. Methanol extract of *M. calabura* exhibited considerable antimicrobial activity against fungal plant pathogens compared to other solvent extracts.

Further, the methanol extract of *M. calabura* root were subjected to column chromatography for the purpose of isolating purified antimicrobial compounds. The methanol crude extract were separated into 23 fractions in column chromatography. The fraction 21 obtained exhibited higher antimicrobial activities against the tested plant pathogens as compared to other fractions. The maximum inhibitory activity in drop diffusion assay is observed for *A. solani* and *F. oxysporum* f.sp. *lycopersici* with a DIZ of 3.2 and 3.0 cm observed against, respectively.

Asiaticoside (the ester glycoside of the triterpene asiatic

acid) and madecassoside (the ester glycoside of the triterpene madecassic acid) are the chief medicinally active compounds of the plant *Centella asiatica* ("Mandukparni") and *Datura stramonium*. These were successfully extracted through column chromatography along with a saponin fraction. The compounds were isolated from other plant constituents by methanol extraction and separated from other terpenes by silica gel column chromatography (Matsuda et al., 2001).

Gas chromatography mass spectrometry identifies the compounds based on their retention indices (determined with reference to a homologous series of normal alkanes), and by comparison of their mass spectral fragmentation patterns. In our present investigation, gas chromatography mass spectrometry studies were carried out for purified antimicrobial fraction obtained from column chromatography

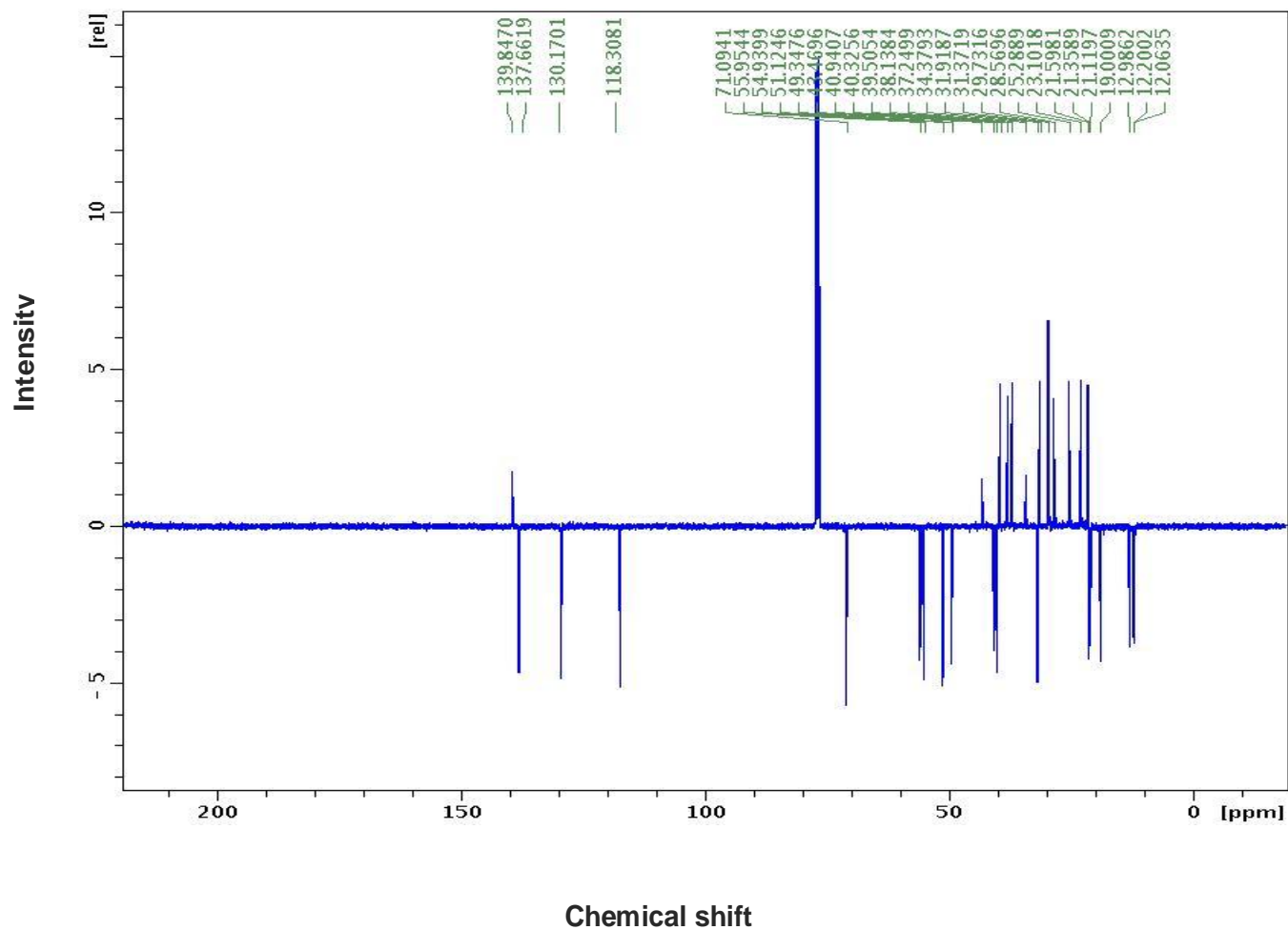


Figure 4. ^{13}C NMR studies of purified antimicrobial fraction F21 from *M. calabura* root extract.

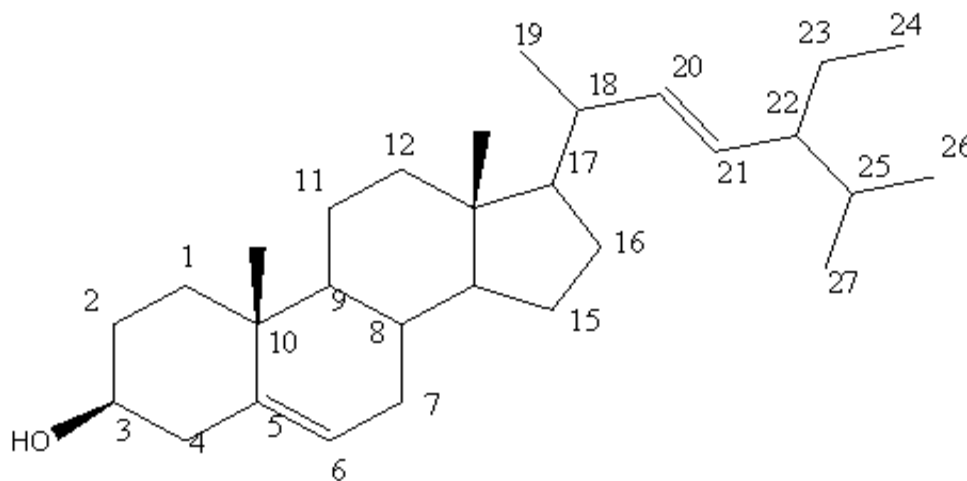
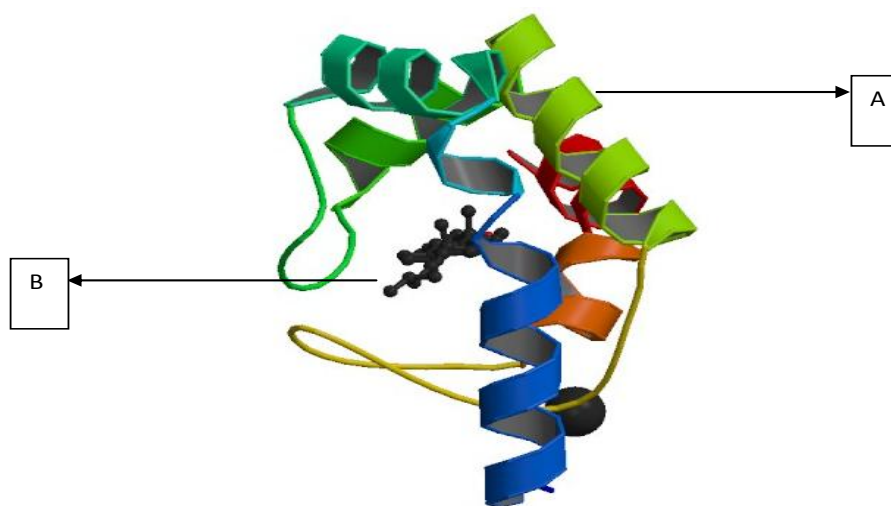


Figure 5. Structure of stigmasterol obtained from *M. calabura* root extract. Molecular weight, 412.70; Formula, $\text{C}_{29}\text{H}_{48}\text{O}$; IUPAC name, (3*S*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-17-[(*E*,2*R*,5*S*)-5-ethyl-6-methylhept-3-en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1*H*-cyclopenta[*a*]phenanthren-3-ol.

Table 4. Induced fit docking results of the compound stigmasterol against the target fungal elicitor Beta-Cryptogein.

Pose	Docking score	Glide energy	Interaction	Distance (Å)
1	-12.59	-42.65	TYR47(O-H...O)	2.70
2	-11.85	-46.75	(O-H...O)TYR87 TYR47(O-H...O)	3.04 3.00
3	-12.04	-44.16	(O-H...O)TYR87 TYR47(O-H...O)	2.94 2.76
4	-12.04	-43.64	TYR47(O-H...O)	2.80
5	-11.43	-44.27	(O-H...O)TYR87	2.91
6	-11.98	-44.55	(O-H...O)TYR87	3.00
7	-11.34	-46.48	(O-H...O)TYR87 TYR47(O-H...O)	2.90 2.88
8	-11.29	-41.80	LYS94(N-H...O)	2.96
9	-11.64	-44.37	(O-H...O)TYR87 TYR47(O-H...O)	3.24 2.99
10	-11.31	-44.89	(O-H...O)TYR87 TYR47(O-H...O)	2.99 2.92
11	-10.78	-43.81	(O-H...O)TYR87	3.27
12	-10.66	-41.73	(O-H...O)TYR87 TYR47(O-H...O) LYS94(N-H...O)	3.09 3.06 3.13
13	-10.63	-45.48	TYR47(O-H...O)	2.72
14	-10.41	-42.01	(O-H...O)TYR87 TYR47(O-H...O)	3.18 2.93



a - Stigmasterol
 b - Fungal elicitor and stigmasterol interaction site.(Try 47 (O-H...O) residue

Figure 6. Molecular docking of stigmasterol (*M. calabura* root) compound with fungal elicitor cprtogein in GLIDE software.

(fraction 21). The mass spectrum of the fraction 21 showed molecular ion peak at m/z 412 associated with the molecular formula $C_{29}H_{48}O$ and consistent with six degrees of unsaturation. The library match of the GC-MS showed that the compound stigmasterol was present in the methanol extract of *M. calabura* root.

1H NMR and ^{13}C NMR spectroscopic studies were carried out for the purified antimicrobial fraction 21 from methanol root extract of *M. calabura*. The spectrum depicts that the isolated fraction is stigmasterol based on their chromatogram pattern. The spectrum of the stigmasterol is confirmed with previous literature collection (Anjoo and Ajay, 2011). The assignment of carbon signals in ^{13}C NMR and the location of substituent in proton NMR and GC-MS suggested that the compound was found to be stigmasterol.

In the present study the IR absorption spectrum showed absorption peaks at 3470cm^{-1} (O-H stretching.); 2867.9cm^{-1} (aliphatic C-H stretching); 1704.6cm^{-1} (C=C absorption peak). These absorption frequencies resemble the absorption frequencies observed for stigmasterol.

Based on the IR spectroscopic studies, the stigmasterol produced absorptions at 3373.6cm^{-1} characteristic of O-H stretching, 2940.7 and 2867.9cm^{-1} is due to aliphatic C-H stretching. Other absorption frequencies include 1641.6cm^{-1} as a result of C=C stretching, however this band is weak; 1457.3cm^{-1} is a bending frequency for cyclic $(CH_2)_n$ and 1381.6cm^{-1} for $-CH_2(CH_3)_2$. The absorption frequency at 1038cm^{-1} signifies cycloalkane. The out of plane C-H vibration of unsaturated part was observed at 881cm^{-1} . Similar kinds of absorption frequencies are observed for fraction 21 which confirms the isolated antimicrobial metabolite as stigmasterol.

The stigmasterol is found as a free or compound in the cell. They are structural components of the lipid core of cell membrane and being precursor of numerous secondary metabolites including plant steroid hormones (Noguchi et al., 2000)

Stigmasterols possess several biological activities, but they also play an important role in defense mechanisms against insects and plant pathogens. An increasing body of evidence indicates that the chemical interactions between plant-pathogenic fungi and higher plants are both complex and highly integrated. For instance, as fungi have developed toxins that increase their virulence on plant tissues, plants have developed a variety of ways to limit the effectiveness of these fungal toxins. Biosynthesis of fungal toxins can, however be blocked *in vitro* by the addition of certain naturally occurring plant metabolites at concentrations inhibitory to fungal growth.

Protein-Ligand Docking in drug discovery and development, the manner in which small-molecule compounds bind or dock with proteins is of utmost importance. Proteins are often the main targets for new drugs; many drug compounds are small molecules that are designed to bind preferentially to specific proteins. Because of this, small molecules need to be design for protein docking;

many bioinformatics tools exist for the analysis of protein-ligand interactions. These tools often fall in the category of computational chemistry. Databases and bioinformatics tools containing genomic, proteomic and functional information have become indispensable in antimicrobial drug research. Many general databases are used in this field, such as Uniport and Protein Data Bank (PDB) (Hughes et al., 1990).

The stigmasterol compound isolated from methanol root extract of *M. calabura* was subjected to protein docking studies with fungal elicitor cryptogein. In the fungal elicitor cryptogein, interaction was observed for TYR-47 with hydrogen bonding interactions only during the induced fit docking in most of the poses. Hence, the docking studies suggest the anti-fungal nature of stigmasterol. The minimum value of the docking score and glide energy obtained for stigmasterol show the binding stability of the compound.

Docking has been used to discover novel ligands for well over 30 targets. The inhibitors discovered were novel, having little similarity to the known ligands. Most initial leads had affinities in the low-micromolar range. The new sulphonamide inhibitors of carbonic anhydrase are the exception; they are much more potent. There has been an efflorescence of new docking methods in the past several years and several docking programs were used in the studies (Shoichet et al., 2002).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

***Angelica gigas* Nakai extract ameliorates the effects of cyclophosphamide on immunological and hematopoietic dysfunction in mice**

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The objective of this study was to develop a treatment for immune and hematopoietic system dysfunction in cancer patients. We induced immunosuppression and hematopoietic dysfunction in mice by injection of cyclophosphamide (CPA) and treated the animals with Korean angelica extract (*Angelica gigas* Nakai), an oriental medicine. Mice were injected with CPA on days 1 and 3 and were orally administered the indicated treatment once daily on days 4 through 8. The animals were analyzed for changes in body weight, spleen weight, hematologic parameters and spleen content of interferon (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-2, IL-7 and IL-10. Treatment of animals with Korean angelica extract ameliorated the effects of CPA on body weight, spleen weight, blood composition and spleen cytokine content. Our results suggest that Korean angelica extract could be an excellent naturally derived therapeutic to treat immunosuppression and hematopoietic dysfunction caused by anticancer agents.

Key words: Korean angelica (*Angelica gigas* Nakai), cyclophosphamide, immune system activation, hematopoietic system activation.

INTRODUCTION

Many anticancer drugs have potentially life-threatening effects on the immune and hematopoietic systems (Ryu et al., 2007). Radiotherapy and chemotherapy are widely used to treat cancer but also have deleterious effects on normal cells. In particular, these treatments may destroy hematopoietic stem cells, thereby inducing severe side effects, such as anemia and leukopenia. As these blood cells are derived from hematopoietic stem cells, the patients can be at higher risk for viral or bacterial infections (Vadhan-Raj, 2009; Wang et al., 2002). Therefore, increasing attention is being paid to combining chemotherapy with treatments that stimulate the immune and hematopoietic systems. In this regard, several studies

have investigated naturally occurring compounds that possess antioxidant activity and that stimulate the immune systems with relatively few side effects (Ryu and Kim, 2005). In particular, many polysaccharides are known to activate cells of the immune system. β -Glucan is a polysaccharide extracted from yeast cell walls that has been reported to have immunostimulatory effects *in vivo* and *in vitro* (Estrada et al., 1997). β -Glucan is a water-soluble fiber that is highly viscous at low concentrations. The consumption of β -glucan as a food and food additive has gradually increased since it was shown to have anticancerous, cholesterol lowering, antioxidant, immunostimulatory and skin regenerative effects (Bobek

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and Galbavy, 2001; Delatte et al., 2001).

In particular, one study reported that *Saccharomyces cerevisiae*-derived β -glucan activates human immune cells and stimulates the production of interferons and interleukins, thereby preventing the proliferation and recurrence of cancer (Ohno et al., 2001). Red ginseng, which is widely consumed in Korea, Japan and China, has been shown to have many nutritional and pharmacological properties, including anti-aging and anticancer effects, and the demand for red ginseng and other oriental medicines has increased (Kong et al., 2008). Red ginseng has been shown to mediate its anticancer effects in rats by influencing many aspects of the immune system, including stimulating natural killer (NK) cells and increasing cell-mediated immunity and antibody-dependent cytotoxicity (Lee et al., 1997). Many other substances extracted and purified from oriental medicines have been shown to be bioactive, including alkaloids, quinoids, terpenoids, polysaccharides, proteins, lipids, steroids, enzymes and vitamins (Han, 1996). Korean angelica (*Angelica gigas* Nakai) has been shown to stimulate blood flow and hematopoiesis and has long been used in Korea and China to treat blood clots and to cure many diseases. A previous study reported that Korean angelica was commonly prescribed for the treatment of woman's diseases and anti-inflammatory, analgesic and anti-thrombolytic properties (Yoon et al., 2007). Korean angelica means 'naturally returning to its own place'. In other words, when blood is congested because of weakened blood circulation, Korean angelica helps in circulating the blood by recovering the circulation energy (Son et al., 2003).

Given the fact that the immune system of human body is highly associated with blood, it is anticipated that Korean angelica could have effects on the immune and hematopoietic systems. We are therefore interested in whether Korean angelica can stimulate the immune and hematopoietic systems when combined with anticancer treatments. Cyclophosphamide (CPA) is a DNA alkylating agent and replication inhibitor that is frequently used as an anticancer agent, either alone or in combination with other anticancer agents. In humans, doses higher than 120 mg/kg cause serious damage to the hematopoietic and immune systems and leads to a notable reduction of leukocytes (Angulo et al., 2000). As part of our investigation into the potential medicinal effect of Korean angelica extract in cancer patients, we examined its effect on immunosuppression and hematopoietic dysfunction in male *crl:CD1* (ICR) mice treated with CPA.

MATERIALS AND METHODS

Experimental animals and diet

Four-week-old male *crl:CD1* (ICR) mice (Orient Bio, Seongnam, South Korea), which are widely used for safety and efficacy testing, were acclimated for 7 days in polycarbonate cages (4 mice/cage) kept at $22 \pm 2^\circ\text{C}$ and ~50 to 60% relative humidity. Animals were

maintained on a 12 h light/dark cycle and were provided animal diet (Samyang, Cheonan, Korea) and drinking water *ad libitum* (Lee et al., 2004). Body weights were measured a total of 8 times at the same time every day, starting the day before CPA injection and ending on the day of sacrifice. These animal experiments were monitored and approved by the Laboratory Animal Ethic Committee of Konkuk University (Seoul, South Korea).

Experimental groups

After the adaptation period, 32 mice (body weight 25.00 ± 0.30 g) were randomly assigned into 4 groups: (1) the normal control group was administered distilled water only, (2) the negative control group was injected with CPA and treated with distilled water, (3) the positive control group was injected with CPA and treated with β -glucan, and (4) the experimental group was injected with CPA and treated with Korean angelica extract (Table 1).

Preparation of Korean angelica extract and β -glucan

Korean angelica was purchased from Ewhadang (oriental medicine hospital) which is located in Kyungdong-market; also the market is well known as a traditional medicine market in Korea. Kim Hyung Min, the oriental medicine doctor of Ewhadang, botanically authenticated Korean angelica. Korean angelica, 8 g, was boiled with 1,300 ml of distilled water for 2.5 h and then filtered through gauze. The filtrate was centrifuged at $8,000 \times g$ for 15 min and the supernatant was filtered again. The filtrate was evaporated to dryness (Rotavapor R-200; Büchi, Flawil, Switzerland) and freeze-dried to yield 2.4 g of powder. The mouse dose was calculated to be 1 mg/25 g body weight, based on the dose for a 60 kg adult (Lee et al., 2005). Korean angelica powder was dissolved in distilled water and 1 mg in 250 μl was orally administered daily for 5 days starting from 24 h after the second injection of CPA. β -Glucan (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in distilled water and administered at 1 mg/kg (25 $\mu\text{g}/250 \mu\text{l}$) on the same schedule (Soltys and Quinn, 1999).

Induction of immunosuppression

Immunosuppression was induced by intraperitoneal injection with 2 doses of CPA (Sigma-Aldrich) in distilled water at 125 mg/kg (3.125 mg per mouse). CPA was administered on days 1 and 3 and mice were then treated with distilled water, Korean angelica or β -glucan on days 4 to 8 (Jung et al., 2009). The normal control group received an intraperitoneal injection of the same volume of sterilized distilled water.

Hematological analysis

On the day of sacrifice, approximately 1 ml of blood was drawn from the mouse heart using a syringe. The blood was placed in tubes containing ethylenediaminetetraacetic acid (EDTA) (Vacutainer; BD Biosciences, Franklin Lakes, USA) and mixed immediately. The blood was then analyzed on an automatic blood analyzer (XE 2100 D; Sysmex, Kobe, Japan) to quantify red blood cells (RBCs), hematocrit, hemoglobin, platelets, white blood cells (WBCs), S-neutrophils, lymphocytes, monocytes, eosinophils and basophils.

Splenic cytokine analysis

On the day of sacrifice, a portion of the spleen (15 mg) was removed and washed twice with phosphate-buffered saline (PBS).

Table 1. Experimental designs.

Group	No. of mice treated	Pretreatment	Composition of treatments
Normal control group	8	None	Basic diet+distilled water
Negative control group	8	CPA	Basic diet+distilled water
Positive control group	8	CPA	Basic diet+ β -glucan
Experiment group	8	CPA	Basic diet+Korean angelica extract

CPA: cyclophosphamide.

Cell lysis buffer (1 ml) from a Mammalian Cell Lysis Kit (Sigma-Aldrich) was added and the tissue was incubated for 15 min on a plate shaker (Shaker 35; Labnet, Woodbridge, USA). The tissue was homogenized at 4°C using a needle and the homogenate was centrifuged at 12,000 rpm and 4°C for 10 min. The supernatant was removed and analyzed for the presence of interferon (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-10, IL-2 and IL-7. Microtiter filter plates were prewetted by placing 200 μ l of wash buffer into each well. The plate was sealed and shaken on a plate shaker for 10 min at room temperature (RT). The wash buffer was aspirated and 25 μ l of standards, controls or assay buffer was added to the appropriate wells. Assay buffer (25 μ l) was added to the sample wells and 25 μ l of RPMI 1640 culture medium (Welgene, Daegu, South Korea) matrix solution was added to the background, standard and control wells. Sample supernatant (25 μ l) diluted 1:1 in assay buffer was added to the appropriate wells. The beads were vortexed and 25 μ l was added to each well. The plates were sealed, covered and agitated on a plate shaker overnight at 4°C or for 2 h at RT. The fluid was then gently aspirated and the plate was washed twice with 200 μ l/well of wash buffer. The excess buffer was removed, the wells were blotted and 25 μ l of detection antibody solution was added to each well. The plates were sealed and incubated on a plate shaker for 1 h at RT. Streptavidin-phycoerythrin (25 μ l) was added to each well and the plates were sealed and incubated on a plate shaker for 30 min at RT. The contents were removed and the wells washed twice with 200 μ l/well wash buffer. Excess buffer was removed and 150 μ l of sheath fluid was added to all wells. The beads were resuspended on a plate shaker for 5 min and the plate was analyzed with a Luminex 200 (Luminex, Austin, USA). The median fluorescence intensity (MFI) data were analyzed using a 5-parameter logistic or spline curve-fitting method to calculate the cytokine concentrations.

Statistical analysis

All results are presented as the mean \pm standard deviation. Statistical differences between groups were analyzed using Duncan's multiple range test.

RESULTS

Weight gain and the ratio of spleen weight to body weight

Over the 8 days of the experiment, animals in the normal control group and the negative control group gained 5.263 ± 0.276 and 1.563 ± 0.172 g of body weight between days 0 and 8, respectively. Both the positive control group treated with β -glucan and the experimental group treated with Korean angelica extract also gained

Table 2. Body weight gain after oral treatment of mice.

Group	Body weight gain (g)
Normal control	5.263 ± 0.276
Negative control	$1.563 \pm 0.172^{###}$
Positive control	$3.6 \pm 0.237^{**}$
Experiment	$3.075 \pm 0.317^{**}$

Each value was represented as mean \pm standard deviation of 8 mice. $^*p < 0.05$ and $^{###}p < 0.01$, significantly different from the normal control group; $^{**}p < 0.01$, significantly different from the negative control group.

weight 3.6 ± 0.237 and 3.075 ± 0.317 g, respectively (Table 2). In the present study, we also observed a noticeable decrease of spleen weight in CPA-treated mice (0.109 ± 0.035 g) compared with the normal control group (0.166 ± 0.035 g) (Table 3). The spleen weights of the positive control group (0.170 ± 0.035 g) and the group treated with Korean angelica extract (0.151 ± 0.022 g) were similar to the weight of the normal control group.

Changes in hematological parameters

The terminal blood samples were examined using an automatic blood analyzer. The red blood cell (RBC) count in the CPA-treated group ($7.11 \pm 0.49 \times 10^6/\mu$ l) was significantly decreased compared with the untreated animals ($8.05 \pm 0.18 \times 10^6/\mu$ l). In contrast, the RBC count in the groups treated with β -glucan and Korean angelica extract were significantly increased ($7.73 \pm 0.29 \times 10^6/\mu$ l and $7.54 \pm 0.44 \times 10^6/\mu$ l, respectively) compared with the negative control group (Table 4). Similar trends were observed for the hematocrit, hemoglobin and platelet measurements. In the negative control groups, these were $39.64 \pm 2.61\%$, 12.11 ± 1.27 g/dl and $1081 \pm 322.26 \times 10^3/\mu$ l, respectively which were significantly lower than the normal control group ($47.935 \pm 3.77\%$, 14.41 ± 0.79 g/dl and $1394.88 \pm 205.15 \times 10^3/\mu$ l, respectively). The hematocrit, hemoglobin and platelet readings for the β -glucan positive control group were $42.31 \pm 2.06\%$, 12.96 ± 0.68 g/dl, and $1642.13 \pm 301.68 \times 10^3/\mu$ l and for the experimental group, $43.20 \pm 2.07\%$, 12.98 ± 0.37 g/dl and $1618.25 \pm 196.63 \times 10^3/\mu$ l, respectively. Thus, the animals treated with β -glucan and Korean angelica extract

Table 3. Changes in spleen weight after oral treatment of mice.

Group	Absolute weight (g)	Relative weight (% of body weight)
Normal control	0.166±0.035	0.581±0.135
Negative control	0.109±0.035 [#]	0.418±0.126 [#]
Positive control	0.170±0.035*	0.615±0.126*
Experiment	0.151±0.022*	0.557±0.091*

Each value was represented as mean ± standard deviation of 8 mice. [#]p < 0.05, significantly different from the normal control; *p < 0.05, significantly different from the negative control.

Table 4. Changes in hematological parameters after oral treatment of mice.

Group	RBC (10 ⁶ /μl)	HCT (%)	Hb (g/dl)	Platelet (10 ³ /μl)
Normal control	8.05±0.18	47.935±3.77	14.41±0.79	1394.88±205.15
Negative control	7.11±0.49 ^{##}	39.64±2.61 ^{##}	12.11±1.27 ^{##}	1081±322.26 [#]
Positive control	7.73±0.29**	42.31±2.06*	12.96±0.68*	1642.13±301.68**
Experiment	7.54±0.44*	43.20±2.07**	12.98±0.37*	1618.25±196.63**

Each value was represented as mean ± standard deviation of 8 mice. [#]p < 0.05 and ^{##}p < 0.01, significantly different from the normal control; *p < 0.05 and **p < 0.01, significantly different from the negative control. RBC, red blood cell; HCT, hematocrit; Hb, hemoglobin.

Table 5. Changes in leukocyte counts after oral treatment of mice.

Group	WBC (10 ³ /μl)	LYM (%)	NEU (%)	MONO (%)	EOS (%)	BAS (%)
Normal control	2.91±0.49	72.1±7.11	14.3±15.32	1.54±0.31	11.2±6.10	1.56±0.74
Negative control	1.49±0.55 [#]	42.9±5.90 ^{##}	37.4±4.01	5.61±1.69	6.8±3.96 [#]	0.83±0.57
Positive control	2.76±1.07*	47.6±5.40*	43.3±11.34	5.38±0.81	11.8±3.38*	1.79±1.00**
Experiment	2.50±1.06*	49.1±4.39*	37.7±7.43	5.90±1.24*	12.2±4.49*	1.83±0.65**

Each value was represented as mean ± standard deviation of 8 mice. [#]p < 0.05 and ^{##}p < 0.01, significantly different from the normal control; *p < 0.05 and **p < 0.01, significantly different from the negative control. WBC, white blood cells; NEU, neutrophils; LYM, lymphocytes; MONO, monocytes; EOS, eosinophils; BAS, basophils.

extract showed significant recovery in the hematological parameters compared with the negative control group.

Similar to the effects on the red blood cell (RBC) counts, mice treated with CPA showed significant reductions in their white blood cell (WBC) counts compared with animals in the normal control group and this effect was significantly alleviated by treatment with β-glucan or Korean angelica extract (Table 5). Total WBC counts were 1.49 ± 0.55 × 10³/μl in the negative control group, 2.91 ± 0.49 × 10³/μl in the normal control group, 2.76 ± 1.07 × 10³/μl in the β-glucan-treated group and 2.50 ± 1.06 × 10³/μl in the Korean angelica extract-treated group. The percentage of WBC made up by lymphocytes was also markedly decreased in the CPA-treated animals compared to the normal controls (42.9 ± 5.90% vs. 72.1 ± 7.11%). On the other hand, these levels were modestly but significantly increased by treatment with β-glucan or Korean angelica extract (47.6 ± 5.40 and 49.1 ± 4.39%, respectively). The same effect

was observed for the percentage of eosinophils and basophils. The percentage of eosinophils were 11.2 ± 6.10, 6.8 ± 3.96, 11.8 ± 3.38, 12.2 ± 4.49% and the percentage of basophils were 1.56 ± 0.74, 0.83 ± 0.57, 1.79 ± 1.00 and 1.83 ± 0.65% for the normal controls, negative controls, β-glucan-treated and Korean angelica extract-treated groups, respectively. CPA-induced immunosuppression is known to be accompanied by a reduction in WBC, RBC and platelet counts (Son et al., 2003).

Changes in splenic cytokines

In the present study, spleens from CPA-treated mice contained lower levels of IL-2 than spleens from the normal group (5.813 ± 0.528 pg/ml vs. 8.226 ± 1.409 pg/ml). In contrast, spleen IL-2 levels were significantly higher in the β-glucan-treated group (7.499 ± 0.904 pg/ml)

and the Korean angelica extract-treated group (7.376 ± 1.911 pg/ml). We found that the IL-7 content of spleens from the negative control group of mice was much lower than that of the normal control group (0.585 ± 0.307 pg/ml vs. 1.238 ± 0.887 pg/ml), whereas the IL-7 levels in the positive control group and the experimental group were similar to those in the normal control group (1.249 ± 0.587 and 1.418 ± 0.748 pg/ml, respectively). Korean angelica extract had a particularly notable effect on the spleen IL-7 content. Similar trends were observed for spleen IL-10 levels. IL-10 levels were lower in the CPA-treated group than the normal control group (15.692 ± 2.538 pg/ml vs. 49.928 ± 17.62 pg/ml) and the effect of CPA was significantly reversed by treatment with β -glucan and Korean angelica extract (45.035 ± 13.934 and 32.793 ± 5.067 pg/ml, respectively). In this study, we found that the IFN- γ content in the spleens of CPA-treated mice was substantially lower than that of the normal control group (41.748 ± 15.806 pg/ml vs. 149.575 ± 77.888 pg/ml). In contrast, IFN- γ levels were significantly higher in the β -glucan-treated and Korean angelica extract-treated groups (172.605 ± 26.889 and 108.924 ± 44.369 pg/ml, respectively) than in the negative control group. TNF- α levels were also reduced in the spleens of the negative control mice compared with the normal control group (4.216 ± 0.889 pg/ml vs. 7.435 ± 2.227 pg/ml). However, whereas the CPA-induced reduction was completely reversed by β -glucan treatment (7.66 ± 0.873 pg/ml), animals treated with Korean angelica extract showed only a slight elevation in spleen TNF- α content (5.615 ± 1.729 pg/ml).

DISCUSSION

Administration of CPA to mice induced weight loss, as has been shown in a previous study (Sadeghi et al., 2008). Similar trends were observed in the present study. Over the 8 days of the experiment, body weight of negative control group was much less than normal control group. In contrast, mice administered Korean angelica extract or β -glucan had significantly increased body weights compared with the negative control group. The spleen, thymus and lymphatic system are important components of the immune system. Previous studies have reported a reduction of spleen and thymus weights in mice following intraperitoneal injection of CPA (McKallip et al., 2002; Miyauchi et al., 1990). Similar trends were observed in our study.

CPA, a nitrogen mustard alkylating agent, is used to treat lymphoma, leukemia and solid cancers, and bone marrow toxicity is a side effect of the immunosuppression. In turn, bone marrow toxicity leads to further hematopoietic dysfunction, which manifests as leukopenia, anemia and thrombocytopenia. Especially, thrombocytopenia has the risk for bleeding problems, which needs platelet transfusions (Busse et al., 1997; Vadhan-Raj, 2009).

Recently, *Artemisiae Capillaris Herba* aqueous extracts and *Panax ginseng* have been reported as effective oriental medicine for the treatment of immunosuppression and hematopoietic dysfunction (Jung et al., 2009; Lee et al., 1997). In the present study, we observed that total WBC, RBC and platelet counts were markedly reduced in the negative control group. However, the reductions were greatly improved by treatment with Korean angelica extract, which was especially effective in preventing the reduction in platelet counts. Vitamin B12, vitamin A and nicotinic acid are major components of Korean angelica extract. The roots contain coumarin derivatives such as decursin, decursinol, nodakenin and umbelliferone, as well as volatile compounds such as β -eudesmol, α -pinene, limonene and elemol and organic acids such as ferulic acid. Among these components, vitamin B12 and decursin have been reported to reconstitute bone marrow, improve hematopoiesis and increase hemoglobin levels in patients with pernicious anemia. Decursinol has also been reported to have preventive and therapeutic effects on hematopoietic dysfunction caused by cancer chemotherapies (Swanson et al., 1995).

Taken together, these data show that the active ingredients in Korean angelica significantly improved the hematopoietic parameters of CPA-treated mice. Beta glucan has been widely used to ameliorate immune response suppressed by anti-cancer agents. This project was to check if *Angelica gigas* used widely as an oriental medicine and has ameliorating activity, in which beta-glucan was used as a positive control. The result indicates that a mixture of *A. gigas* demonstrated a similar effect as beta glucan so that a single major compound isolated from *A. gigas* mixture could have better effect (at least similar effect) than beta glucan and be obtained in the future. Therefore, the results strongly suggest that Korean angelica has potential effect as a naturally derived immunostimulant for the immunosuppression induced by CPA. The spleen is a secondary immune organ and supports initial immune responses against exogenous antigens (Meloni et al., 1994). Accordingly, splenocytes produce multiple cytokines, including interferon (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-2, IL-7 and IL-10 (Fry and Mackall, 2002).

CPA dramatically reduces T lymphocyte counts and inhibits cytokine secretion (Xun et al., 1994). Therefore, we next examined the effect of Korean angelica extract on the cytokine content of spleens from CPA-treated mice (Table 6). The health of late-stage leukemia patients can be improved by administering IL-2, which stimulates cytolytic T cell-mediated killing of cancer cells resistant to anticancer agents (Meloni et al., 1994). IL-7 is an important cytokine that contributes to differentiation of T and B lymphocytes (Fry and Mackall, 2002), stimulation of thymocytes, activation of NK cells and lymphokine-activated lymphocytes and production of IL-4 and IFN- γ (Albina et al., 1989). IL-10 has a number of effects on many cell types, including T cells, B cells, macrophages

Table 6. Splenic cytokine levels after oral treatment of mice.

Group	IL-2 (pg/ml)	IL-7 (pg/ml)	IL-10 (pg/ml)	INF-gamma (pg/ml)	TNF-alpha (pg/ml)
Normal control	8.226±1.409	1.238±0.887	49.928±17.62	149.575±77.888	7.435±2.227
Negative control	5.813±0.528 ^{##}	0.585±0.307 [#]	15.692±2.538 ^{##}	41.748±15.806 ^{##}	4.216±0.889 ^{##}
Positive control	7.499±0.904 ^{**}	1.249±0.587 [*]	45.035±13.934 ^{**}	172.605±26.889 ^{**}	7.66±0.873 ^{**}
Experiment	7.376±1.911 ^{**}	1.418±0.748 [*]	32.793±5.067 ^{**}	108.924±44.369 ^{**}	5.615±1.729

Each value was represented as mean ± standard deviation of 8 mice.

[#]p < 0.05 and ^{##}p < 0.01, significantly different from the normal control; ^{*}p < 0.05 and ^{**}p < 0.01, significantly different from the negative control.

and monocytes. For instance, IL-10 suppresses the secretion of IL-2 and IFN- γ by T cells and inhibits the synthesis of IL-1, TNF- α , IL-6, IL-8 and colony stimulating factors by monocytes (Van der Poll et al., 1996). IFN- γ is produced by CD8+ T cells, CD4+ Th1 cells and NK cells, and has effects on numerous cell types, including B cells, T cells, NK cells and macrophages (Isaacs, 1995). TNF- α is a typical proinflammatory cytokine produced by many cell types, including splenocytes, and is known to play a crucial role in T lymphocyte differentiation and activation of cell-mediated immunity (Samira et al., 2004).

Collectively, our results showed that CPA significantly reduced the splenic content of TNF- α , IL-2, IL-7, IFN- γ and IL-10. Notably, treatment of mice with Korean angelica extract significantly reversed the CPA-induced suppression of these cytokines. Korean angelica is generally used as a medicine that activates blood flow in oriental medicine. The main components of Korean angelica, including decursin and volatile compounds are thought to facilitate blood flow through the coronary arteries, promote the production of RBCs and elevate the phagocytic capacity of monocytes and macrophages as part of their anti-inflammatory, analgesic and immunostimulatory activities. These compounds possess antioxidant, radioprotective and hepatoprotective properties, and are effective therapies for leukemia (Swanson et al., 1995). Thus, it seems likely that these active components of Korean angelica extract might also be responsible for the effects on cytokine levels in our experiments.

In this study, Korean angelica has an ameliorative effect with immunosuppression by CPA. Considering its extract as a mixed compound compared with β -glucan, it has more effect as a single compound isolated from Korean angelica. These results suggest that Korean angelica has potential as a treatment for patients with immune dysfunction to anticancer therapy. Furthermore, based on its effects on hematological parameters, Korean angelica extract could be an excellent candidate for development as a hematopoiesis-stimulating agent.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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