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ANALYTICAL, BIOLOGICAL AND PHYLOGENETIC STUDIES OF *CLERODENDRUM PHLOMIDIS* LINN. F. LEAVES

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ABSTRACT

Clerodendrum phlomidis Linn. f. (Lamiaceae) is a well known medicinal plant used for the treatment of nervous disorders and mental diseases. Hence, the study was planned to investigate the various extracts, fractions and reported compounds of *C. phlomidis* leaves for acetylcholinesterase (AChE) inhibition and cytotoxic activity. Crude polyamine fraction (CPF) showed highest percentage inhibition (67.38%) of AChE. The unsaponified petroleum ether fraction of methanol extract (UPFME) showed high lethality with an LC_{50} value of 1130.70 µg/mL against brine shrimp lethality bioassay. β-carotene was identified for the first time from *C. phlomidis* leaves which was further quantified by high performance thin layer chromatography (HPTLC). The β-carotene content of the leaves was found to be 0.0218 %w/w. DNA analysis of *C. phlomidis* leaves was carried out which was then compared to the phylogenetic tree of Lamiaceae. The cladistic analysis of *C. phlomidis* confirmed its phylogenetic origin from Africa and not from Asia.

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INTRODUCTION

It is well-known that Alzheimer's Disease (AD) is characterized by degradation of the cholinergic system together with alteration of glutamatergic and serotoninergic receptors [1]. Acetylcholinesterase (AChE) inhibitors increase the availability of acetylcholine in central cholinergic synapses and are the most promising currently available drugs for the treatment of AD [2]. Cholinesterase inhibition is not only the mainstay treatment for AD but also considered as promising strategy for the therapy of dementia, myasthenia gravis and Parkinson's disease. Thus regulating the activities of AChE has become an important research focus.

Clerodendrum phlomidis Linn. f. (Lamiaceae) is a well known medicinal plant in *Ayurveda* and *Siddha* system of medicine which constituted more than 50 indigenous drug formulations. Its popular uses include the treatment of nervous disorder and mental diseases [3]. Hence, this study was planned to investigate the various extracts, fractions and reported compounds of *C. phlomidis* leaves for acetylcholinesterase inhibition and cytotoxic activity. β-carotene was identified for the first time from *C. phlomidis* leaves which was quantified by using high performance thin layer chromatography (HPTLC). DNA analysis of *C. phlomidis* leaves was carried out and was then compared to the phylogenetic tree of Lamiaceae.

MATERIALS AND METHODS

Chemicals, reagents, and solvents

Acetylthiocholine iodide (ATCI), tris hydrochloride (Tris-HCl), acetylcholinesterase (AChE) was procured from Sigma Aldrich, Bengaluru. Ellman's reagent (DTNB), bovine serum albumin was procured from Himedia Laboratories Pvt Ltd, Mumbai. Brine shrimp eggs were purchased from Ocean Star International Inc., Snowville, UT, USA. All other chemicals, reagents and solvents were obtained from SD Fine Chemicals (Mumbai, India) and were of analytical grade. The phylogenetic study was carried out at University of Tasmania, Australia.

Plant material

Leaves of *C. phlomidis* were collected from out-fields of Trichy, Tamil Nadu, India. Authenticated plant material (Voucher specimen: HDT/CP/08-09/MKM/15) was deposited in the Herbarium of Medicinal Plants, Pharmacy Department, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India.

Quantification of β-carotene

A Camag TLC system equipped with Camag Linomat V an automatic TLC sample spotter, Camag glass twin trough chamber (20 X 10 cm) were used for the analysis. The quantitative evaluations of the plates were performed with CAMAG scanner 3 (win CATS 4.0 integration software). Accurately weighed 5 g of the coarse powder of *C. phlomidis* leaves was extracted with methanol (4 x 50 mL) under reflux (30 min each time) on a water bath. The combined extracts was filtered, concentrated and made up to 50 mL with methanol. The working solutions were prepared by appropriate dilution of the stock solution with methanol. A stock solution of standard β-carotene (100 µg/mL) was prepared in methanol. Standard β-carotene in the range of 100 to 500 ng spot^{-1} was applied on TLC plate for preparation of calibration curve. 5 µL of the sample solution was applied in triplicate on the TLC plate and developed with the mobile phase n-hexane: benzene (9:1, v/v). Densitometric scanning was performed in the absorption-reflection mode at 445 nm with a computerized Camag TLC scanner. Peak areas were recorded and the quantity of β-carotene present in the sample solution was calculated using the calibration curve [4].

Amino acid identification

The preliminary chemical tests of *C. phlomidis* leaf extract showed the presence of amino acids. Hence, their identification was carried out by comparative thin layer chromatography (Co-TLC).

Preparation of extracts and fractions

Air-dried leaves (10 g) of *C. phlomidis* were extracted by centrifuging with 3 x 100 ml of water, 50% methanol and methanol at 1000 g for 15 min. The supernatants were combined, evaporated to dryness under reduced pressure on rotary evaporator and further dried in desicator to yield aqueous extract (AE), 50% methanol extract (50%ME) and methanol extract (ME) respectively. Air-dried leaves (10 g) were grounded and extracted with methanol in Soxhlet apparatus for 48 h. The extract was evaporated to dryness under reduced pressure on rotary evaporator (Rotavapor, Buchi) and further dried in desiccator to yield methanol extract. The methanol extract was further fractioned by centrifuging with 3 x 100 ml of petroleum ether (60-80 °C) at 1000 g for 15 min. The supernatants were combined, evaporated to dryness under reduced pressure on rotary evaporator and further dried in desiccator to yield petroleum ether fraction of methanol extract. The insoluble residue was designated as residual fraction of methanol extract (RFME). The petroleum ether fraction of methanol extract was saponified and the unsaponified matter was designated as unsaponified petroleum ether fraction of methanol extract (UPFME). A crude polyamine fraction (CPF) was prepared as per reported method [5].

Reported and isolated compounds

The previously reported compounds like β-sitosterol, lupeol, L-dopa, adrenaline and β-carotene from leaves [6, 7] were procured from Himedia Laboratories Pvt. Ltd., Mumbai, India or Sigma Chemicals, Bangalore, India. Clerosterol and clerosterol palmityl ester were isolated in Herbal Drug Technology Laboratory, Pharmacy Faculty, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. [8].

Acetylcholinesterase micro plate inhibition assay

25 µl of substrate, 15mM of ATCI in Millipore water, 125 µl of 3mM DTNB in buffer C (50mM Tris–HCl, pH 8, 0.1M NaCl, 0.02M $MgCl_2.6H_2O$), 72.5 µl of buffer B (50mM Tris–HCl, pH 8, 0.1% bovine serum albumin) and 2.5 µl of sample solution dissolved in DMSO were added into 96-well plates. 25 µl of 0.22 U/ml AChE in buffer B were added to the above wells and the absorbance were read in a microplate reader (BioRad 680XR, France) at 405 nm after three minutes. The percentage inhibition was calculated by comparing the rates for the samples to the control [9]. The experiment was performed in triplicate. The extract and fraction were tested at 4000 µg/mL and the compounds at 400 µg/mL.

Cytotoxicity study

Cytotoxicity was studied by brine shrimp lethality bioassay as per the method of Meyer et al. (1982) [10]. Brine shrimps (*Artemia salina*) were hatched from eggs in a conical shaped vessel (1L), filled with artificial seawater (prepared using sea salt 38 g per liter and adjusted to pH 8.5 using 1N NaOH) with constant aeration for 36 h at room temperature (20±5 °C) under light. After hatching, the active nauplii, free from egg shells were collected from brighter portion of the hatching chamber were used for the assay. Ten nauplii were drawn through a glass capillary and placed in vials each containing 4.5 mL of brine solution (24% of NaCl in water). 0.5 mL of water/extracts/fractions/compounds were added into the vials and maintained at room temperature (20±5 °C) under light. The number of surviving nauplii after 24 h was counted. Each sample was studied in six vials along with a control group upto 2000 µg/mL. Extracts/fractions/compounds were dissolved in minimum volume of DMSO and made up with water. The concentration of DMSO was studied as a vehicle control. The % lethality was determined from the number of surviving nauplii in control and sample using the below mentioned formula;

$$\% \text{ lethality} = \frac{NSNC - NSNS}{NSNC} \times 100$$

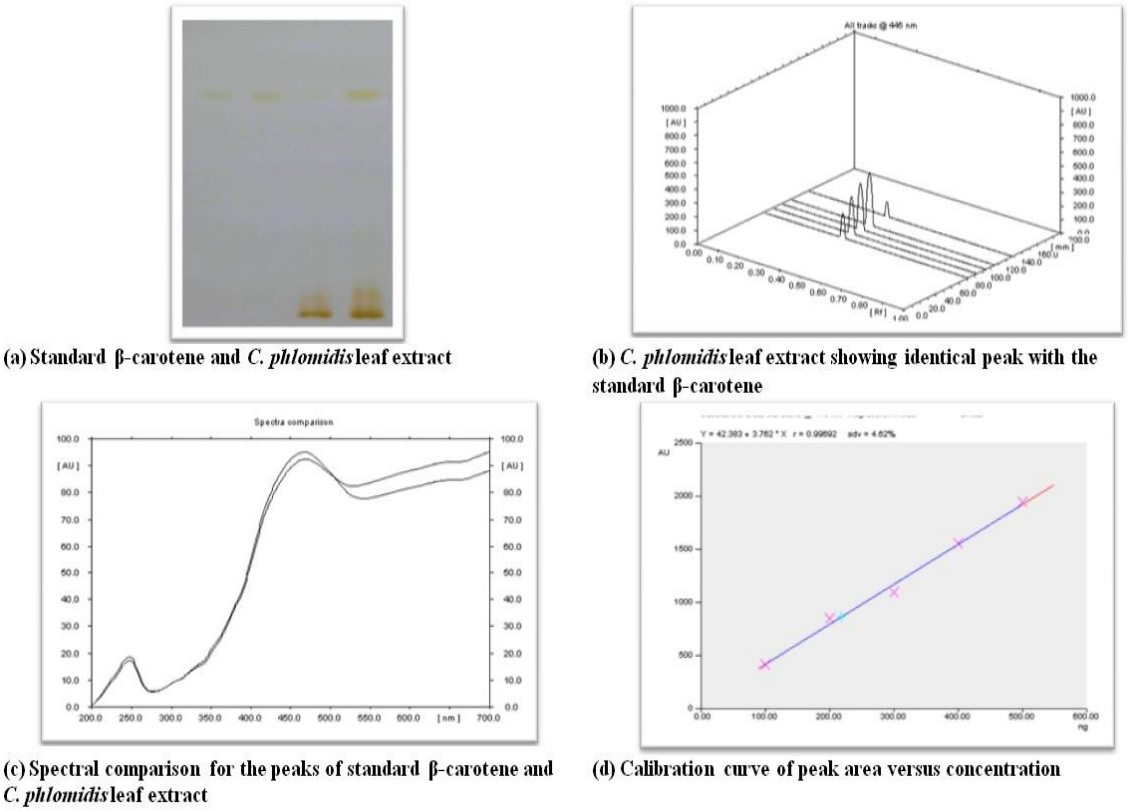
NSNC is the number of surviving nauplii in control and NSNS is the number of surviving nauplii in sample. LC_{50} values were calculated from percentage lethality Vs concentration best-fit line graph.

Phylogenetic study

DNA sequence for internal transcribed spacer (ITS) region of *C. phlomidis* was carried out. Total DNA was extracted from silica gel dried leaf tissue and the 5.8S nr DNA and flanking ITS regions were amplified using the polymerase chain reaction (PCR) with primers ITS 5 and ITS 4. The amplification conditions were 97 °C for 1 min, 48 °C for 1 min and 72 °C for 45 seconds increasing by 4 seconds/cycle over 40 cycles. Single-stranded DNA was produced by including 10 µL of double-stranded DNA in a second 100 µL reaction mixture containing only one of the two primers. Twenty-five cycles of PCR were required for the single-stranded amplifications. Single-stranded PCR products were sequenced with TAQuence (Amersham, Arlington Heights, Illinois), using 32P dATP, in accordance with the recommendations of the manufacturer. The sequencing reactions were primed using the ITS 5 and ITS 4 primers externally, and ITS 3 and ITS 2 internally. Both strands of DNA were sequenced. To overcome the band compressions in the gels, reactions containing 7-deaza-2´-deoxyguanosine 5´-triphosphate were run in addition to reactions containing dGTP (2´-deoxyguanosine 5´-triphosphate). Sequences were aligned using the DNA sequence alignment program Clustal V followed by visual inspection (GenBank database). The new sequence were also aligned by eye to the aligned ITS sequences from Steane et al. (1997) and Steane et al. (1999) [11, 12]. Alignment gaps were scored as separate binary characters following the 'simple gap coding' method. Parsimony analyses were carried out using PAUP ver. 4.0b10 (Phylogenetic Analysis Using Parsimony – a computational phylogenetics program for inferring evolutionary trees) [13] using heuristic searches with 100 replicates, each with ten random order entry starting trees, Tree Bisection and Reconnection (TBR) branch swapping, and saving multiple trees at each step Multi-labeled trees on (MUL-trees). All data sets were bootstrapped 250 times following DeBry and Olmstead (2000) [14] using 10 random order entry starting trees per replicate, TBR branch swapping and MUL-trees off. The consensus analysis was carried out by combining the sets of trees that had been obtained in previous analyses and semistrict consensus calculations.

RESULTS AND DISCUSSION

The leaf extract of *C. phlomidis*, when subjected to TLC showed the presence of β-carotene peak (Figure 1a). A comparison of the spectral characteristics of the peaks for standard and that of the sample further confirmed the presence of β-carotene in leaf (Figure 1b and 1c). Good resolutions with symmetrical and reproducible peaks were obtained. The peak area versus concentration plots was found to be linear in the range of 100-500 ng $spot^{-1}$ for β-carotene (Figure 1d). The regression equation and correlation coefficient for β-carotene indicated good linearity (Table 1). The β-carotene content of the leaves was found to be 0.0218 %w/w (Table 1).



(a) Standard β-carotene and *C. phlomidis* leaf extract

(b) *C. phlomidis* leaf extract showing identical peak with the standard β-carotene

(c) Spectral comparison for the peaks of standard β-carotene and *C. phlomidis* leaf extract

(d) Calibration curve of peak area versus concentration

Figure 1. Showing the TLC plate, chromatogram, spectral analysis and calibration curve for β-carotene in *C. phlomidis* leaf extract.

Table 1. Linearity regression data for quantification of β-carotene in *C. phlomidis* leaf extract.

Parameter	β-carotene
R_f	0.40
Dynamic range (ng spot^{-1})	100-500
Equation	y=42.383+3.762x
Slope	3.762
Intercept	42.383
Linearity (correlation coefficient)	0.997
Specificity	Specific
Amount of compound quantified* (% w/w)	0.0218

*plant dry weight basis.

The co-TLC study showed the presence of tyrosine, phenylalanine, alanine, valine, leucine, isoleucine, glutamic acid and threonine (Figure 2a). Polyamines are widely distributed in plants, microorganisms, and animal tissues. Wide spread polyamines include putresine, spermidine, agmatine and spermine [15]. The co-TLC of *C. phlomidis* extract showed the presence of numerous polyamine bands (Figure 2b).

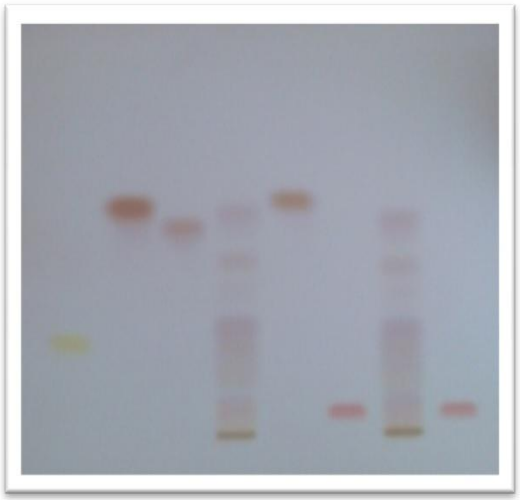


Figure 2a. Showing the Co-TLC of amino acids and *C. phlomidis* extract

Figure 2b. TLC showing the presence of polyamines in *C. phlomidis* extract

Figure 2. Showing the TLC of amino acids and polyamines in *C. phlomidis* leaf extract.

AChE inhibition was measured using the analogue acetylthiocholine iodide, which is converted to thiocholine in the presence of AChE. The reaction of thiocholine with the chromogenic substrate dithionitrobenzoic acid (DTNB) leads to the formation of 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate (yellow color), which absorbs strongly at 405 nm. CPF showed highest percentage inhibition (67.38%) of acetylcholinesterase (Table 2). Polyamines inhibit AChE on substrate-concentration-dependent activation and is similar to that by Na^+, K^+, Ca^{2+}, Mg^{2+} and certain quaternary and bisquatemary amines [16]. L-dopa, lupeol, adrenaline and clerosterol showed 62.23, 60.88, 55.84 and 53.9% of AChE inhibition respectively (Table 2), which coincides with reported literatures [17-20]. The 58.46% inhibition by AE may be due to the presence of polyamines, L-dopa and adrenaline. The ME showed 52.31% inhibition, in spite of lupeol and clerosterol being constituents of the extract. There was no synergistic effect observed. 50%ME, UPFME, RFME, β-sitosterol, β-carotene and clerosterol palmityl ester showed less than 50% inhibition. Percentage inhibition of AChE is directly proportional to the polarity of *C. phlomidis* leaf extract.

Table 2. Percentage inhibition of AChE by extracts/fractions/compounds of *C. phlomidis* leaves.

Extracts/fractions/compounds	% of inhibition
AE	58.46
50%ME	47.66
ME	52.31
UPFME	46.16
RFME	48.16
CPF	67.38
β-sitosterol	28.53
Lupeol	60.88
L-dopa	62.23
Adrenaline	55.84
β-carotene	25.30
Clerosterol	53.93
Clerosterol palmityl ester	35.36

Brine shrimp lethality assay is a rapid, reliable and has been used for over thirty years in cytotoxic, phototoxic, pesticidal, trypanocidal, enzyme inhibition, and ion regulation activities [21]. LC_{50} values < 1500 µg/mL was considered significant, UPFME showed high lethality among extracts and fractions (Table 3). The tested compounds followed the order clerosterol>clerosterol palmityl ester>β-carotene>β-sitosterol in lethality to brine shrimps. Lupeol, L-dopa and adrenaline showed no significant effect till 2000 µg/mL. Brine shrimp lethality bioassay has good correlation with the human solid tumour cell lines [22]. Hence, it can be suggested that the sample UPFME is bioactive with cytotoxic and anti-tumour activity.

Table 3. The lethality effect of *C. phlomidis* leaves on brine shrimps.

Extracts/fractions/compounds	LC_{50} values (µg/mL)
AE	> 2000
50%ME	> 2000
ME	1340.24
UPFME	1130.70
RFME	> 2000
CPF	> 2000
β-sitosterol	750.22
Lupeol	> 2000
L-dopa	> 2000
Adrenaline	> 2000
β-carotene	520.36
Clerosterol	330.71
Clerosterol palmityl ester	450.85

Currently, DNA sequencing is applied to distinguish species, to study phylogenetic relationship, population genetics, systematics and evolution [23]. The ITS rDNA region has become an important locus for the molecular systematic investigation of angiosperms at the interspecific and intraspecific levels. The DNA sequences for ITS of two samples of *C. phlomidis* A and B (Figure 3a & 3b) differed by one base pair, may be one of the samples is polymorphic/heterozygous for that position in the sequence. Figure 4 illustrates the position of *C. phlomidis* relative to other species of Clerodendrum and related genera. In a phenetic study by Stenzel et al. (1988) [24] *C. phlomidis* was placed in a group with other Asian species (clade I). The cladistic analysis shows that *C. phlomidis* belongs to clade II – the African clade (Figure 4). Moreover the cladistic study also showed that *C. phlomidis* is very closely related to *Clerodendrum hildebrandtii* var. *puberula* Verdc., a Tanzanian species. The sequence of *C. phlomidis* was also compared to other sequences on GenBank (GenBank database accession number gb|U77743.1|CBU77743) which showed a close resemblance to *Clerodendrum buchneri*, the other species in Clade II (African).

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>C._phlomidis_A
TGGGAGGATAAAAAACGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGG
ATCATTGTCGAAACCTGCATAGCAGACCGCGAACACGTGTTTAAACAAAT
CGGGGCTGCGGTCTTCTGCGGTCCCCTCATCGCCGGCGTGCGCCAACGCG
TCGCTGTGCGGTCTAACAAAATCGGGCGCGGAATGCGCCAAGGAATACAC
AAAAGAGTGTTCCCCTCCCCAGGGCCCATGTGCGGAGATCGTGGGGAGGT
TGGGATGCCCGTCGTATACAAAAACGACTCTCGGCAACGGATATCTCGGC
TCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGC
AGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCA
TTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCGCCTCCCT
CCACACACAGTGCTGTTGATGGGGGCGGATATTGGCCTCCCGTGCATCAT
TCATGCGCGGCCGGTCCAAATGCAATCCCTCGGTGGCGAAAGTCACGACC
AGTGTTGGTTGAAGTATCAACTCGCGTGCTGTCGTGACACAAGACGTCGT
CCGATCGGGAGTCACTACAGACCCAGTGGCGCATTCACGCATTGCGCCTC
CGACCGCGACCCCAGGTCAGGCGG
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Figure 3a. DNA sequences for ITS of *C. phlomidis.*

>C._phlomidis_B
AACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAAACCTGC
ATAGCAGACCGCGAACACGTGTTTAAACAAATCGGGGCTGCGGTCTTCTG
CGGTCCCCTCATCGCCGGCGTGCGCCAACGCGTCGCTGTGCGGTCTAACA
AAATCGGGCGCGGAATGCGCCAAGGAATACACAAAAGAGTGTTCCCCTCC
CCAGGGCCCATGTGCGGAGATCGTGGGGAGGTTGGGATGCCCGTCGTATA
CAAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAA
CGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATC
GAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTC
TGCCTGGGCGTCACGCATCACGTCGCCTCCCTCCACACACAGTGCTGTTG
ATGGGGGCGGATATTGGCCTCCCGTGCATCATTCATGCGCGGCCGGTCCA
AATGCAATCCCTCGGTGGCGAAAGTCACGACCAGTGTTGGTTGAAGTATC
AACTCGCGTGCTGTCGTGACACAAGACGTCGTCCGATCGGGAGTCACTAC
AGACCCAGTGGCGCATTCACGCATTGCGCCTCCGTCCGCGACCCCAGGTC
AGGCGGGATTACCCGCTGAGTTTAAGCATATC

Figure 3b. DNA sequences for ITS of *C. phlomidis.*

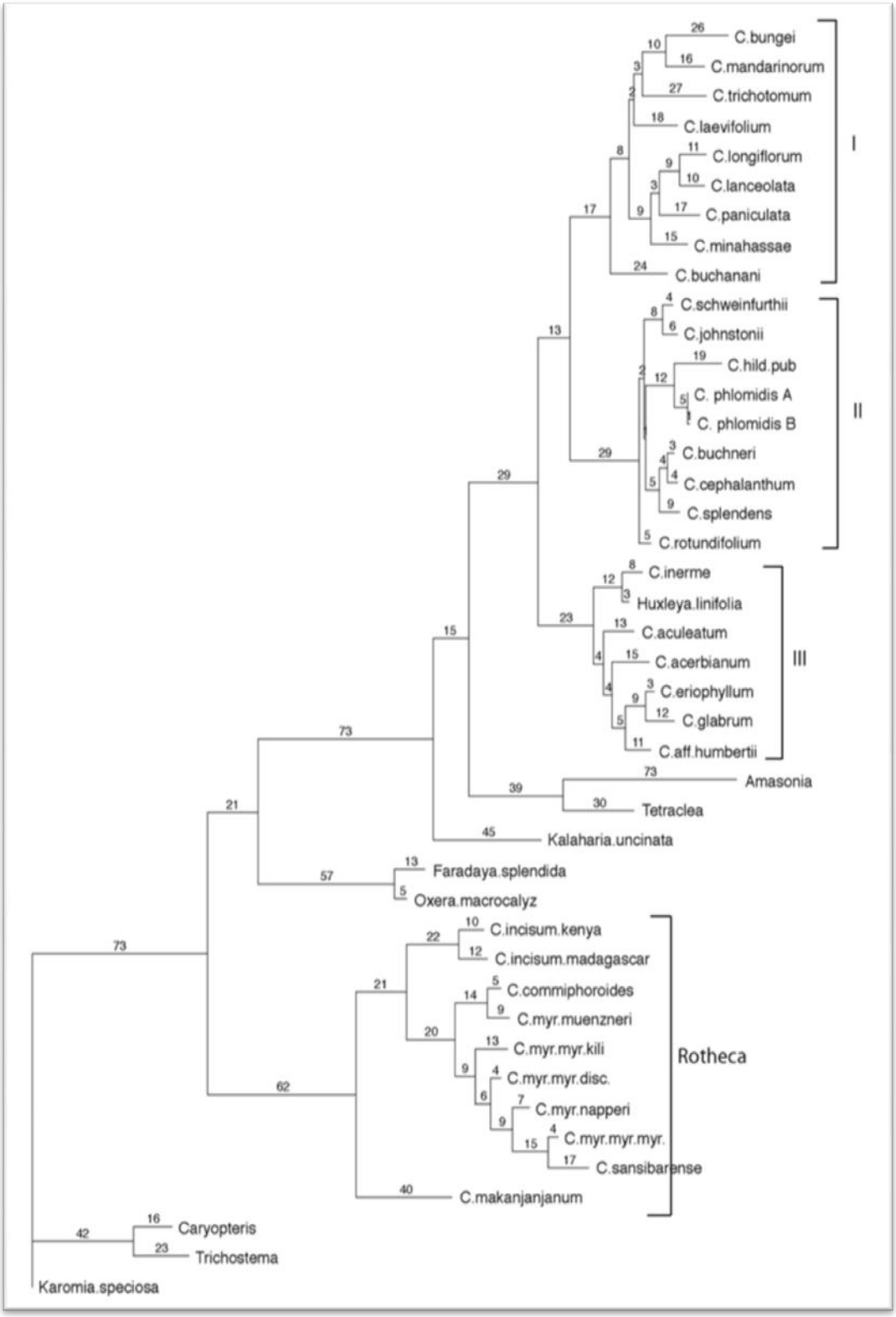

Figure 4. Parsimony tree of *C. phlomidis* from cladistic analysis of ITS sequence data.

C. phlomidis is geographically distributed in India (Andhra Pradesh, Bihar, Diu Island, Gujarat, Haryana, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Orissa, Punjab, Rajasthan, Tamil Nadu, Uttar Pradesh and West Bengal), Pakistan (Baluchistan and Sindh provinces), Sri Lanka, Myanmar and South-east Asia [3] (Figure 5). *C. hildebrandtii* and *C. buchneri* are distributed along the South-east coast of Africa (Mozambique, Tanzania and Kenya) (Figure 5). Hence, it can be concluded that *C. phlomidis* has its phylogenetic origin from Africa and certainly not from Asia. *Clerodendrum phlomidis* is widely distributed in Asia but phylogenetically originated from African species (clade II). This contradiction can only be explained by the sub-merged continent of "Kumari Kandam" (50,000 BCE) that connected South-east Africa, South India and Australia [25, 26].

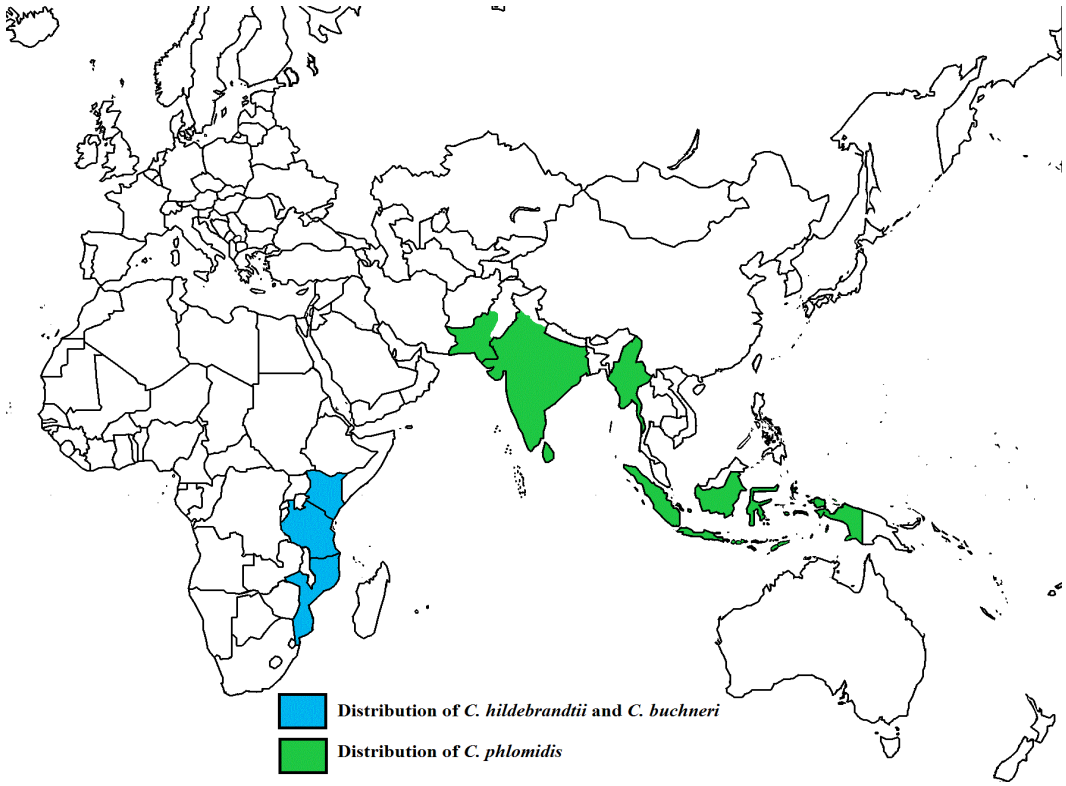

Figure 5. Showing the distribution of *Clerodendrum hildebrandtii*, *C. buchneri* and *C. phlomidis*.

CONCLUSION

The various extracts, fractions and reported compounds of *C. phlomidis* leaves were investigated for acetylcholinesterase (AChE) inhibition and cytotoxic activity. Crude polyamine fraction (CPF) showed highest percentage inhibition of AChE. The unsaponified petroleum ether fraction of methanol extract (UPFME) showed high lethality against brine shrimp lethality bioassay. β-carotene was identified for the first time from *C. phlomidis* leaves which was further quantified by high performance thin layer chromatography (HPTLC). The cladistic analysis of *C. phlomidis* confirmed its phylogenetic origin from Africa and not from Asia.

Recommend future Research

The further investigation of the sub-merged continent of "Kumari Kandam" (50,000 BCE) that connected South-east Africa, South India and Australia would further confirm the origin of *C. phlomidis.*

Authors' Statements

The authors declare that there is no conflict of interests.

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