*Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 27, No. (3–4) : 2025 : 163-168*
*© EM International*
***ISSN-0972-3005***

**DOI No.: http://doi.org/10.53550/AJMBES.2025.v27i03-04.005**
**Journal Home Page:** www.envirobiotechjournals.com

# BIOASSAY GUIDED ISOLATION OF ANGIOTENSIN CONVERTING ENZYME INHIBITOR COMPOUND VERBASCOSIDE FROM POTENTIAL *CLERODENDRUM* SPECIES OF NORTH EAST INDIA

**JANMONI KALITA[1]* AND S. SURESHKUMAR SINGH[2]**

[1]Department of Botany, Behali Degree College, Borgang 784 167, Assam, India
[2]Department of Botany, School of Life Sciences, Manipur University, Canchipur, Manipur 795 003, India

(Received 13 January, 2025; Accepted 29 March, 2025)

***Key words:*** *Angiotensin converting enzyme, Antihypertensive, Angiotensin converting enzyme inhibitor, Clerodendrum, North East India, Verbascoside.*

**Abstract–** Verbascoside isolated from leaf extract of *Clerodendrum inerme* was found as an Angiotensin Converting Enzyme inhibitor for the first time. *In vitro* antihypertensive evaluation of six *Clerodendrum* species and a multi technique based approach comprising of bioassay guided thin layer chromatography, liquid chromatography-mass spectrophotometry and UV spectrophotometry were used to identify the potential Angiotensin Converting Enzyme inhibitor compound, verbascoside from column fractions of *Clerodendrum inerme*. The potent sub fraction F5.2.5 showed maximum of 80% ACE inhibition. LC-MS of crude methanol leaf extract revealed presence of mass peaks with 625.3 m/z [M+H]+ confirming the presence of verbascoside like molecule (exact mass 624.5 m/z) in the sample and most potent sub fraction F5.2.5 displayed the fragmented isotopic mass peaks of Verbascoside (163.2, 325.2, 479.2 and 647.2 m/z [M+H]+) revealing the Angiotensin Converting Enzyme inhibitor of *Clerodendrum inerme* is probably a phenyl glycoside like Verbascoside. The identity of the Verbascoside was confirmed finally with the measurement of absorption maxima (λ-max) and dark brown TLC band of F5.2.5 as compared to the standard reference compound. The result suggests that the antihypertensive effect of *Clerodendrum inerme* may be, at least in part, due to ACE inhibitor effect of verbascoside in sample.

## INTRODUCTION

The treatment and management of hypertension disease is primarily consumption of drugs and medicines which includes Angiotensin Converting Enzyme (ACE) inhibitor, beta blocker, thiazide diuretic and calcium channel blocker. Verbascoside (Acteoside or Kusagin) is an antihypertensive compound which inhibits ACE, Rock II, PDE5 enzymes which are involved in the progression of hypertension in human body. Verbascoside is also known to possess many pharmacological properties like antioxidant, anti-inflammatory, antimicrobial, anti-glycation, anti-hemolytic, anthelmintic, hepato-protective, anti-hypertensive and memory enhancement, etc. (He *et al.,* 2011). The compound is distributed in more than 200 species of several families including Acanthaceae, Bignoniaceae, Lamiaceae, Verbenaceae, Oleaceae etc. (Scogin, 1992; Schlauer *et al*., 2004; Taskova *et al*., 2005). Verbascoside is the main component of a herbal formulation "CHDA Capsules" (G20060728) which is available in the Chinese commercial market as a hepatoprotective health product (Chen *et al.,* 2012). The compound is water soluble and easily diffused into the water when the leaves are boiled.

*Clerodendrum* is a very large and medicinally important genus distributed in tropical and subtropical regions of the world. Some species of the genus have been continuously used by local ethnic communities of North East India either in the form of leaf decoction or leaf infusion for the treatment of various ailments and diseases such as antimicrobial, antihelminthic, anti-inflammatory, antimalarial, antidiabetic, hepatoprotective, indigestion, high blood pressure, fever, asthma, etc. (Das *et al*., 2008; Khan and Yadava, 2010; Sanjem and Gosai, 2006; Tamuli and Saikia, 2004). Various biological activities like antibacterial, antimicrobial, insecticidal, antihypertensive, antioxidant etc. and

major chemical steroids, alkaloids, phenolics etc. have been reported (Srivastava and Patel, 2007). However, little scientific reports are available on evaluation of antihypertensive properties and isolation of verbascoside from important *Clerodendrum* species of North East India.

Angiotensin converting enzyme (ACE) is a peptide hormone that helps in regulating blood pressure and fluid balance in the body. ACE converts angiotensin I to angiotensin II, a potent vasoconstrictor that increases blood pressure (Skeggs *et al.*, 1956). Search for ACE inhibitor compounds has been one of the most important research priorities for development of drugs for the treatment and management of hypertension. Commercial inhibitors of ACE such as captopril, enapril, lisinopril are widely used in the clinic for the treatment of hypertension. Therefore, screening, discovery and development of agents which inhibit the conversion of angiotensin I to angiotensin II is a therapeutic strategy for drug development to treat hypertension.

The present objective was taken to evaluate the antihypertensive property as well as to identify and isolation of verbascoside from leaf extract of potential *Clerodendum* species.

## MATERIALS AND METHODS

### Collection of plant material and preparation of methanol leaf extracts

*In vitro* antihypertensive evaluation by Angiotensin-I Converting enzyme (ACE) inhibition assay was carried out for six medicinally important *Clerodendrum* species namely *C. inerme*, *C. indicum*, *C. japonicum*, *C. philippinum*, *C. serratum*, *C. viscosum* collected from Assam and Arunachal Pradesh of North East India. The collected species were properly identified by consulting identifying keys (Srivastava and Choudhary, 2008). Herbaria of six *Clerodendrum* species were prepared and deposited in Biotechnogy lab, Gauhati University, Assam. Young, tender, disease free leaves of six *Clerodendrum* species were dried, suspended in methanol (ME) and crude ME extracts were screened for antihypertensive assay.

### Angiotensin Converting Enzyme assay (ACE)

### Chemicals and reagents

Angiotensin I converting enzyme (EC 3.4.15.1-0.25U) (A6778), HEPES, Hippuryl-L-Histidyl-L-Leucine (HHL), cyanuric chloride, captopril, boric acid, EDTA, potassium chloride, potassium hydroxide, sodium hydroxide, verbascoside (HWI01068) were purchased from Sigma Aldrich.

### *In vitro* ACE assay

*In vitro* ACE activity was determined by monitoring the transformation from a substrate hippuryl-histidyl-leucine (HHL) to the product hippuric acid (HA) catalyzed by ACE (Schnaith *et al*., 1994; Hurst and Lovell-Smith, 1981).

A volume of 59 µl incubation buffer was taken into a test tube (10x100 mm) and 20ìl of enzyme (10mU) was added followed by mixing gently. The reaction tube was pre-incubated at 37 °C ±1°C for 10 min. After incubation, 96 µl of the substrate (3mM HHL) and 25 µl sample extract (1mg/ml) were added and gently mixed. The mixture was incubated at 37 °C for 30min. The reaction was terminated by adding 1000 µl of stop solution (100mM HEPES-EDTA). 750 µl colour reagent, cyanuric chloride was added followed by vortexing for 30 sec. The mixture was centrifuged at 3000rpm for 10min. 200 µl of the upper supernatant was transferred into 96-well microplate and the absorbance of the yellow colour was measured at 384nm in a spectrophotometer (MultiskanGO, Thermos-Scientific, Finland). Captopril was used as the standard inhibitor of ACE. A positive control reaction without inhibitor and appropriate sample blanks were included for all the samples assayed.

### Enzyme, substrate and buffers preparation

**Assay buffer (188mM boric acid pH 8.3, 1.37M potassium chloride)**: 2.91g of boric acid and 25.63g of potassium chloride dissolved in 200 ml bidistilled water, adjusted to pH 8.3 with 1M potassium hydroxide and dilute to 250 ml with water.

**Enzyme preparation (100mU)**: A stock of 1mg/ml (100 mU) of Angiotensin I Converting Enzyme (ACE) prepared by dissolving in deionized water and kept in -20 °C (refrigerator).

**Substrate solution (6mM of Hippuryl-L-Histidyl-L-Leucine)**: HHL prepared in potassium borate buffer. This solution was prepared freshly before each assay.

**Stop solution (100mM)**: 23.83g of HEPES and 0.93g EDTA dissolved in 800ml bidistilled water, adjusted to pH 9.0 with NaOH 1M and diluted to 1 liter.

**Colour reagent (136mM cyanuric chloride in 1,4 dioxane)**: 12.5g of cyanuric chloride dissolved in

500ml of 1,4dioxane.

**Test sample (1mg/ml)**: 1mg/ml of test sample prepared in DMSO and further diluted with water up to 1ml. DMSO concentration was below 2%.

### Isolation, purification and characterization of bioactive compounds from leaf extract of *C. inerme* species

The crude ME extract of (32g) was suspended in a little amount of water (approx. 20ml) and then subsequently partitioned with three solvents in order of increasing polarity, nhexane (NH), ethyl acetate (EA) and n-butanol (NB).

### Purification of ethyl acetate (EA) extract by silica gel column chromatography

The EA extract (6g) partitioned from crude ME was further purified by using silica gel column chromatography and nhexane (NH), chloroform (CL), methanol (ME) were used as eluent solvents for separation of the EA extract. 6g of sample was mixed with little amount of (0.5g) of silica gel (100-200 mesh). Then vacuum dried to get uniform and fine mixing powder. The collected column fractions (EAF1-F5) were dried and in vitro ACE inhibitor test was performed in all fractions. The active column fraction 5 (EAF5, 2g) was further purified by silica gel column chromatography using NH, CL and ME as eluting solvents. A total of three sub-fractions (EAF5.1, EAF5.2 and EAF5.3) were collected from this process and all were evaluated against ACE for their bioactivity. The most active sub-fraction (EAF5.2) was again purified by silica gel column chromatography using the same eluting solvents. A total of seven (EAF5.2.1-EAF5.2.7) fractions were collected during the separation process. All fractions were properly dried and yields were recorded. *In vitro* ACE test was done for all fractions for their bioactivity.

### Characterization of compound

### Thin layer chromatography (TLC)

EA and its active column fractions F5, F5.2, F5.2.5 were characterized by TLC in the ratio of CL: ME: Water (14.5:5:0.5). Further the chromatogram was recorded after treatment with 5% sulphuric acid and then TLC plates were gradually heated from 60-100 ºC.

### UV-Visible Spectroscopy

The absorption maxima (λ-max) of most inhibited fraction F5.2.5 was recorded on a UV-visible spectrometer at room temperature.

### LC-MS

The crude ME and column fraction F5.2.5 along with standard verbascoside was analyzed with LC-MS in positive electron spray ionization (+ESI) mode. The analysis was done on Agilent 6410 Triple Quad MS-MS. A volume of 10 µl of sample was injected through a Zorbax ODS Analytical C-18 column (5 ml, 4.6x250mm, #880952-702-C18, Agilent) under isocratic mode at a flow rate of 1 ml/min for 20 minutes with water (1% formic acid). The mass spectrum of the isolated compound was recorded in positive ESI mode between 50 to 1000 m/z with a setting of 300 °C temperature and 15 l/min flow rate of gas, nebulizer pressure at 15 psi, capillary voltage at 4000 °C and fragmentation at 135volts.

## RESULTS

### *In vitro* ACE inhibition bioassay

The ME leaf extracts of one species *C. inerme* (CI) have shown significant ACE inhibition properties (65%) as compared to other species. Lowest inhibition was shown by *C.serratum* (CS) 6% followed by *C. viscosum* (CV) 8%, *C. philippinum* (CP) 10% and 11% for *C.indicum* (CIN). There was no inhibition of the enzyme activity *C.japonicum* at a uniform concentration (25ìlH"125ìg/ml) against captopril (CPT, 80%), the standard inhibitor drug of ACE.

Based on the results of screening ACE inhibition assay, *C. inerme* species was chosen for further bioassay and the crude leaf extract was further fractionated into three phases for isolation and identification of ACE inhibitor (Figure 1).

**Fig. 1.** *Clerodendrum inerme* (L.) Gaertn. in natural habitat.

### ACE inhibition properties of crude ME leaf extract and column fractions of *C. inerme*

The results of the ACE inhibition assay of partitioned extracts of crude ME and subsequent column separated fractions are shown in Figure 2a. Among the 3 partitioned ME extracts, NB (17%) and NH (34%) extracts had shown insignificant ACE inhibition activity whereas EA extract showed highest inhibition (65%) as compared to captopril (CAP) 75% inhibition. The first column fractions of EA extracts, EAF1, EAF3 showed low inhibition (27%, 35%), EAF2, EAF4 did not show ACE inhibition while EAF5 revealed highly significant inhibition (75%) of the ACE activity (Figure 2b). Further, among the 3 sub-fractions of EAF5, two fractions EAF5.2 (76%) and EAF5.3 (70%) showed maximum inhibition as compared to EAF5.1 with minimum inhibition (28%) (Figure 2c). Finally, four sub-fractions (EAF5.2.1 to EA5.2.4) of EAF5.2 did not show ACE inhibition and the remaining three sub-fractions, the highest ACE inhibition was shown by EAF5.2.5 (80%) followed by EAF5.2.6 (42%) and EAF5.2.7 (35%) as compared to 86% inhibition by captopril (Figure 2d).

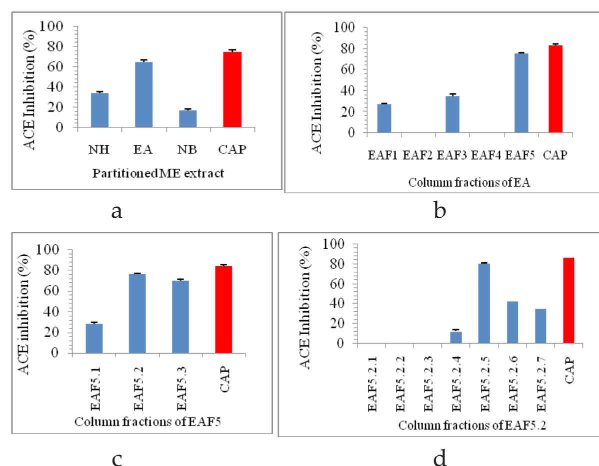


**Fig. 2.** ACE inhibition assay of crude methanol leaf extract of *C.inerme*. (a) NH, EA and NB partitioned extracts (b) column fractions of EA extract (c) column fractions of EAF5 (d) column fractions of EAF5.2

### TLC profiles of crude extract and column fractions

The TLC chromatogram of EA, EAF5, EAF5.2 and last purified fraction EAF5.2.5 showed a dark brown band (*rf*= 0.72) after spray-drying of the plate with 5% sulphuric acid and this band was found closely similar to the band of the standard compound verbascoside (V) (Figure 3)

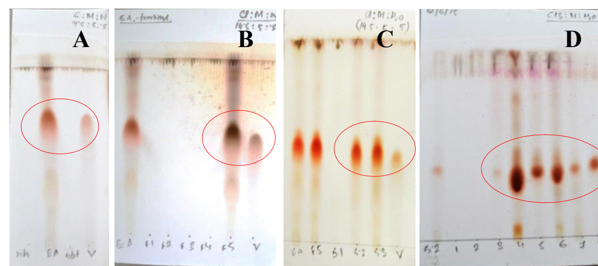


**Fig. 3.** TLC profiles of crude leaf extract and column fractions from *C. inerme* after spray-rying of 5% sulphuric acid (A to D)

### LC-MS analysis

The LC-MS spectrum of +ESI scan of crude ME extract showed presence of a precursor ion peaks of 625.3 m/z $[M+H]^+$ along with 624.2 m/z $[M+H]^+$ (Figure 4a). The LC-MS spectrum of +ESI scan of most purified column fraction of F5.2.5 displayed the isotopic mass peaks (fragmented ions) of 163.1, 325.1, 479.2, and 642.3 m/z [M+H]+. When ionization in positive mode, verbascoside produces these fragmented ions as m/z 642.2$[M+NH_4]^{1+}$, 479.2[M-Rha residue]$^{1+}$,325.1[M-HT-Rha]$^{1+}$, and 163 [M-HT-Rha-Glc]$^{1+}$ which have been widely reported in published literature (Calis *et al*., 1990; Ying *et al*., 2004) (Figure 4b,c) .

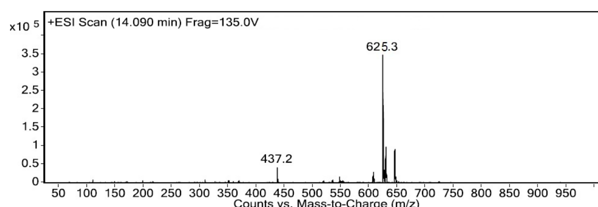


**Fig. 4a.** LC-MS [+ESI] spectra of crude ME of *C. inerme.*

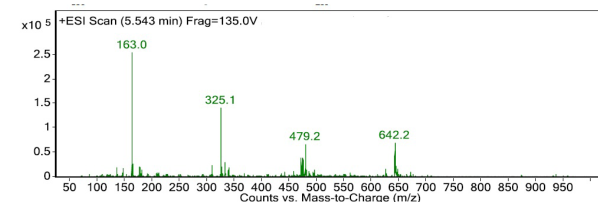


**Fig. 4b.** LC-MS [+ESI] spectra of EA column fraction F5.2.5

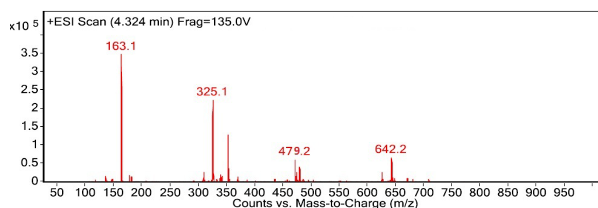


**Fig. 4c.** LC-MS [+ESI] spectra of standard verbascoside

**Absorption spectrum of purified column fraction**

The UV-visible scan of the purified column fraction (F5.2.5) displayed four absorption peak ranges at 216-218nm, 244-246nm, 288-290nm and 330-333 nm when dissolved in water and ME. These peaks were found to be similar to absorption peaks of the standard compound, verbascoside dissolved in ME (Figure 5).

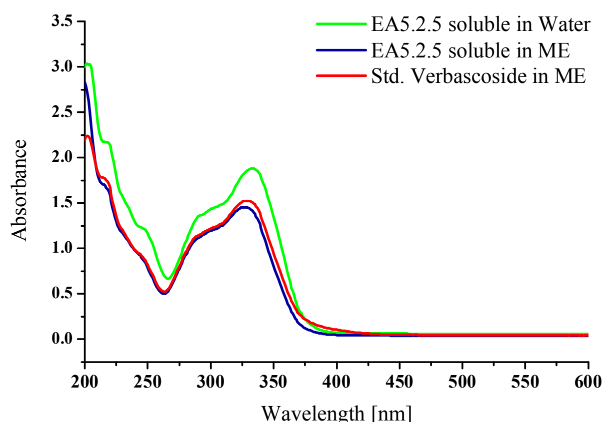


**Fig. 5.** Absorption spectra (λ-max) of purified column fraction (F5.2.5) as compared to standard compound verbascoside

## DISCUSSION

The isolation and identification of verbascoside as a potential ACE inhibitor was reported for the first time from leaf extract of *C. inerme*. Previously, verbascoside was isolated as a phytochemical from the same species but its relationship was not established with hypertension disease (Fauvel,1989; Tran and 2022). In this investigation a new multi-technique integrated approach of bioassay, chromatography, spectrometry and mass spectrometry for isolation and identification of bioactive compound, verbascoside (acteoside) from crude plant extract was studied. In this approach, every column fraction which shows above 50% ACE inhibition was considered as a criterion to select the sample for analysis of TLC profile, absorption maxima as well as LC-MS for isolating and identifying the principle compound. The use of verbascoside as an external standard compound with previously known to occur in the plant helped us to find the target compound in the entire approach, particularly during TLC, spectrometry and LC-MS analysis.

The presence of the dark brown TLC band with *rf* value 0.72 corresponding to that of the standard reference compound, verbascoside in every sample with significant ACE inhibition helped in deciding the fate of the sample for next experimental step. The result of the TLC profile was also validated by analysis of LC-MS spectra. The positive electron spray ionization-mass spectra of most potent sub fraction F5.2.5 also displayed the associated isotopic mass peaks of verbascoside (163.2, 325.2, 479.2 and 647.2 m/z [M+H]+) revealing the ACE inhibitor compound of *C. inerme* is probably a phenylglycoside like verbascoside. Verbascoside is a natural, water-soluble polyphenol glycoside in which the phenylpropanoid caffeic acid and the phenylethanoid hydroxytyrosol form an ester and an ether bond respectively, to the rhamnose part of a disaccharide. Fragmentation of verbascoside occurs when the molecule loses a hexose sugar or a caffeoyl moiety. This process is a type of chemical dissociation that can be studied using mass spectrometry. The identity of the verbascoside was confirmed finally with the measurement of absorption maxima (λ-max) of F5.2.5 as compared to the standard reference compound. The presence of four absorption peaks in the UV region of the spectra indicates the presence of verbascoside as main compound in the sample. The presence of these absorption peaks have also been reported for verbascoside isolated from *Brandisia hancei* and *Plantago psyllium* (Zhou *et al*., 2004; Li *et al.*, 2005). The occurrence and distribution of verbascoside and its an alogs among the members of genus *Clerodendrum* has been well reported but its relation with hypertension was not established (He *et al*., 2011; Shrivastava and Patel, 2007). A recent study showed verbascoside and its analogs from ethanolic extract of stem parts of *Clerodendrum trichotomum* and pheolic compounds isolated from leaf extracts of *Clerodendrum volubile* with significant *in vitro* ACE inhibition properties (Adefegha and Oboh, 2016; Kang *et al*., 2013). Chen *et al*., (2012) also reported antioxidative and antihypertensive properties of verbascoside and its analogs using both *in vitro* and *in vivo* bioassays.

## CONCLUSION

*C. inerme* has been found rich source of verbascoside and further studies on cellular toxicity using *in vitro* cell lines, *in vivo* animal models may lead to development of herbal drug from the leaf of the plant for treatment and management of hypertension at every home with or without other

therapeutic medicines. Verbascoside can be used as pharmaceutical standard and may also be marketed commercially since the cost of the pure compound is very high (INR 40,000/10mg). A semi-preparative HPLC or counter-current high performance chromatography (CCHPLC) based purification of the compound obtained from *C. inerme* in the present study may give high purity of the compound.

**ACKNOWLEDGEMENTS**

Authors are grateful to Guwahati Biotech Park, IIT Guwahati, Assam and JNU, New Delhi for providing instrument facilities. DBT sponsored Institutional Biotech Hub, Gauhati University is highly acknowledged for carry out laboratory work.

**Conflict of interest** – None

**REFERENCES**

Adefegha, S.A. and Oboh, G. 2016. Antioxidant and inhibitory properties of *Clerodendrum volubile* leaf extracts on key enzymes relevant to non-insulin dependent diabetes mellitus and hypertension. *J. Taibah Univ. Sci.* 4(10): 521-533.

Calis, I., Basaran, A.A., Saracoglu, I., Sticher, O. and Ruedi, P. 1990. Phlinosides A, B and C, three phenylpropanoid glycosides from Phlomis *linearis*. *Phytochem.* 29(4): 1253-1257.

Chen, C., Lin, Y., Chien, M., Hou, W. and Hu, M. 2012. Antioxidant and antihypertensive activities of acteoside and its analogs. *Bot. Stu.* 53: 421-429.

Das, A.K., Dutta, B.K. and Sharma G.D. 2008. Medicinal plants used by different tribes of Cachar district, Assam. *Indian J. Tradit. Knowl.* 7: 446-454.

Fauvel, M.T., Gleye, J. and Andary, C. 1989. Verbascoside: A Constituent of *Clerodendrum inerme. Planta Med.* 55(6):577.

He, J., Hu, X., Zeng, Y., Li, Y., Wu, H.Q. and Qiu, R.Z. 2011. Advanced research on acteoside for chemistry and bioactivities. *J. Asian Nat. Prod. Res.* 13: 449-464.

Hurst, P.L. and Lovell-Smith, C.J. 1981. Optimized assay for serum Angiotensin-Converting Enzyme activity. *Clin. Chem.* 27(12): 2048-2052.

Kang, D.G, Lee, Y.S., Kim, H.J., Lee, Y.M., Lee, H.S. 2013. Angiotensin converting enzyme inhibitory phenylpropanoid glycosides from *Clerodendron trichotomum*. *J. Ethnopharmacol*. 89(1): 151-154.

Khan, H.M. and Yadava, P.S. 2010. Antibiabetic plants used in Thoubal district of Manipur, Northeast India. *Indian J. Tradit. Knowl.* 9: 510-514.

Li, L., Tsao, R., Liu, Z., Liu, S., Yang, R. and Young, J.C. 2005. Isolation and purification of acteoside and isoacteoside from *Plantago psyllium* L. by high-speed counter-current chromatography. *J. Chromatogr. A.* 1063: 161-169.

Sajem, A.L. and Gosai, K. 2006. Traditional use of medicinal plants by the Jaintia tribes in North Cachar Hills district of Assam, Northeast India. *J. Ethnobiol. Ethnomed.* 2: 33.

Schlauer, J., Budzianowski, J., Kukulczanka, K. and Ratajczak, L. 2004. Acteoside and related phenylethanoid glycosides in *Byblislini* flora Salisb. plants propagated *in vitro* and its systematic significance. *Acta Soc. Bot. Pol.* 73:9-15.

Schnaith, E., Beyrau, R., Biickner, B., Kleinb, R.M. and Rick, W. 1994. Optimized determination of angiotensin I-converting enzyme activity with hippuryl-L-histidyl-Lleucine as substrate. *Clin. Chim. Acta.* 227: 145-158.

Scogin, R. 1992. The distribution of acteoside among angiosperms. *Biochem. Syst. Ecol.* 20(5): 477-480.

Shrivastava, N. and Patel, T. 2007. *Clerodendrum* and healthcare: An overview – Part II Phytochemistry and Biotechnology. *Med. Aromat. Plant Sci. Biotechnol.* 1(2): 209-223.

Skeggs, L., Kahn, J. and Shumway N. 1956. The preparation and function of the hypertension-converting enzyme. *J. Exp. Med.* 103(3): 295-299.

Srivastava, N. and Patel, T. 2007. *Clerodendrum* and Healthcare: An overview. *Med. Aromat. Plant Sci. Biotechnol.* 1: 209-223.

Srivastava, R.C. and Choudhary, R.K. 2008. Species diversity and economic importance of the family Verbenaceae in Arunachal Pradesh. *Bull. Arun. Prad. Res.* 24(1&2): 1-21.

Tamuli, P. and Saikia, R. 2004. Ethno-medico-botany of the Zeme tribe of North Cachar Hills district of Assam. *Indian J. Tradit. Knowl.* 3: 430-436.

Taskova, R.M., Gotfredsen, C.H. and Jensen, S.R. 2005. Chemotaxonomic markers in Digitalideae (Plantaginaceae). *Phytochem.* 66: 1440–1447.

Tran, M.T. and Tran, H.V. 2022. Verbascoside extracted from *Clerodendrum inerme*: A natural monomer for the fabrication of a sensitive electrochemical Cu(II) sensor. *J. Chem. Res.* 46(3): 1-7.

Ying, T., Luhua, Z., Lin, Z., Guangji, W. and Binren, X. 2004. Separation and identification of 20chemical constituents in the traditional Chinese medicinal preparation shenbao tablet by LC–ESI-MS3. *J. Chromatogr. Sci.* 42: 177-183.

Zhou, L.Y., Hua, Y., Wei, N. and Chen, C.X. 2004. Phenylpropanoid glycosides from *Brandisia hancei*. *Acta Bot. Yunnan.* 26(3): 349-354.