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2012 Oct-Dec Volume 5 Issue 4

Review Articles

PECTIN BASED FORMULATIONS FOR BIOMEDICAL APPLICATIONS: A REVIEW	1-7
R. K. MISHRA, A. K. BANTHIA AND A. B. A. MAJEED	
A REVIEW ON MEDICINAL PLANTS FOR NEPHROPROTECTIVE ACTIVITY	8-14
MOHANA LAKSHMI. S, USHA KIRAN REDDY. T AND SANDHYA RANI. KS	
A REVIEW ON DIABETES MILLETUS AND THE HERBAL PLANTS USED FOR ITS TREATMENT	15-21
MOHANA LAKSHMI. S, SANDHYA RANI. K. S, USHA KIRAN REDDY.T	

Research Articles

TRESCRIBING TREND OF ANTIHITTERTENSIVE DROGS IN HITTERTENSIVE AND DIABETIC HITTERTENSIVE FATIENTS	22-23
K.KOUSALYA, SOWMYA CHIRUMAMILLA, S.MANJUNATH, S.RAMALAKSHMI, P.SARANYA, D.CHAMUNDEESWARI PERIPLANATA AMERICANA - A CARRIER OF MULTI RESISTANT LISTERIA SPECIES	24-27
SHINI ZACHARIA, ASHA PETER, JYOTHIS MATHEW, P.C RAVINDRAN ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF ETHANOLIC EXTRACT OF PARTHENIUM HYSTEROPHORUS LINN.	28-31
KHUSHBU PANDEY, PRAMOD K. SHARMA, RUPESH DUDHE TARGET LEVEL ANALYSIS OF ANTIOXIDANT ACTIVITY OF COSTUNOLIDE AND EREMANTHIN ISOLATED FROM COSTUS	32-35
SPECIOSUS DAISY.P, SUVEENA. S AND R. CECILY ROSEMARY LATHA	
ACUTE AND SUBACUTE TOXICITY STUDIES OF <i>KODI PAVALAM CHUNNAM</i> IN RODENTS V. VELPANDIAN, S. PREMA, ASHWINI ANJANA, J. ANBU	36-41
SYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITIES OF SOME 2-PYRAZOLINE DERIVATIVES B. DIPANKAR, P. PANNEERSELVAM, B. ASISH	42-46
EFFECT OF NUTS (PISTACHIO OR ALMONDS) CONSUMPTION ON LIPID PROFILE OF HYPERCHOLESTEROLEMIC RATS	47-53
IN VITRO SCREENING OF ANTIMICROBIAL ACTIVITY OF WRIGHTIA TINCTORIA (ROXB.) R. BR.	54-58
MOORTHY K., APARNA ARAVIND., PUNITHA T., VINODHINI R., SURESH M. AND THAJUDDIN N. ACUTE TOXICITY STUDY OF EXTRACTS OF EICHHORNIA CRASSIPES (MART.) SOLMS	59-61
P.LALITHA, SHUBASHINI.K.SRIPATHI AND P.JAYANTHI EFFICACY AND SAFETY EVALUATION OF SESA OIL VS COCONUT OIL IN DIFFERENT HAIR & SCALP AILMENTS:	62-64
PROSPECTIVE, OPEN LABEL, RANDOMIZED COMPARATIVE STUDY MADHURI SHRIVASTAVA	
ANTIDIARRHOEAL ACTIVITY OF ETHANOLIC EXTRACTS OF RUTA GRAVEOLENS LEAVES AND STEM PINKEE PANDEY, ARCHANA MEHTA AND SUBHADIP HAJRA	65-68
STUDY OF THE ANTIHYPERLIPIDEMIC, ANTIOXIDATIVE AND ANTIATHEROGENIC ACTIVITY OF AEGLE MARMELOS LINN. IN RABBIT RECEIVING HIGH FAT DIETS	69-72
WARNAMONI DAS, ABDUL HAKIM, AJAY MITTAL DISSOLUTION RATE ENHANCEMENT OF ATORVASTATIN, FENOFIBRATE AND EZETIMIRE BY INCLUSION COMPLEX WITH	73-76
B-CYCLODEXTRIN ANKIT AIMERA, SHRIKALP DESHPANDE, SEIAL KHARADI, KINIAL RATHOD, KEYUR PATEL AND PRANAV PATEL	
ASSESSMENT OF SYNERGISTIC CAPACITIES OF SULBACTUM WITH A CARBAPENEM	77-80
SAYAN IT MURHERJEE, SHASWATT CHART, T.R. PAL, MUSFIQUA MOURERJEE, SURHEN DAS AND SUJATA G.DASTIDAR <u>SYNTHESIS, ANTIMICROBIAL AND ANTI-INFLAMMATORY ACTIVITIES OF 3-(1-SUBSTITUTED PHENYL-1H-TETRAZOL-</u> 5 VI DRVBIDINE DEBINATIVES	81-84
SHINY GEORGE, SANJAY VARMA. P, INDOORI SURESH & SHANMUGAPANDIYAN.P	
ANTIOXIDANT ACTIVITY OF ETHANOLIC EXTRACT OF MARANTA ARUNDINACEA .L TUBEROUS RHIZOMES NISHAA.S, VISHNUPRIYA.M, SASIKUMAR.J.M, HEPHZIBAH P CHRISTABEL, GOPALAKRISHNAN.V.K	85-88
ANTIBACTERIAL ACTIVITY OF SOME SELECTED SEAWEEDS FROM THE GULF OF MANNAR COAST, SOUTH INDIA D.RADHIKA, C.VEERABAHU, R.PRIYA	89-90
A VALIDATED RP-HPLC METHOD FOR THE ESTIMATION OF DIOSGENIN FROM POLYHERBAL FORMULATION CONTAINING TRIBULUS TERRESTRIS LINN.	91-94
PANKAJ K. GUPTA, DHEERAJ H. NAGORE, VINOD V. KUBER AND SURESH PUROHIT ANTIOXIDANT ACTIVITIES AND CYTOTOXIC EFFECTS OF WHOLE PLANT AND ISOLATED CULTURE OF ARTEMISIA AUCHERI	95-98
BOISS. SORMEH CHAREHMATROSSIAN, VU POPOV MAHLACHA CHORBANLL AND SHILA SAFAFIAN	
ECBOLIN A: A BIOACTIVE COMPOUND FROM THE ROOTS OF ECBOLIUM VIRIDE (FORSSK.) ALSTON	99-101
K FRANCINA CECILIA, R RAVINDHRAN, V DURAIPANDIYAN EVALUATION OF PHYTOCHEMICAL CHARACTERISTICS AND ANTIMICROBIAL ACTIVITY OF PLEUROTUS FLORIDA	102-106
MUSHROOM MENAGA, D., MAHALINGAM, P.U., RAJAKUMAR, S. AND AYYASAMY P.M	
THE EFFECT OF LIGHT, TEMPERATURE, PH ON STABILITY OF BETACYANIN PIGMENTS IN BASELLA ALBA FRUIT S. K. RESHMI, K. M. ARAVINDHAN AND P. SUGANYA DEVI	107-110
ANTIOXIDANT ACTIVITY OF RUMEX VESICARIUS L. AT THE VEGETATIVE STAGE OF GROWTH EL-BAKRY, A.A. MOSTAFA, H.A.M. AND EMAN, A. ALAM	111-117
IN VITRO ANTHELMINTIC ACTIVITY OF FICUS BENGHALENSIS LINN. LEAVES EXTRACTS	118-120
ANTIOXIDANT ACTIVITY OF N-PHENYLBENZOHYDROXAMIC ACID	121-124
DEEPESH KHARE, BHARATI VERMA AND RAMA PANDE MOLECULAR UNDERSTANDING AND INSILICO VALIDATION OF TRADITIONAL MEDICINES FOR PARKINSON'S DISEASE	125-128
MUTHUSAMY JEYAM, GANGA R RAJ KARTHIKA, VASUDEVAN POORNIMA AND MANOHARAN SHARANYA ANTIPYRETIC AND ANALGESIC EFFECT OF METHANOLIC EXTRACT OF DIFFERENT PARTS OF ABROMA AUGUSTA LINN	129-133
SUTAPA DAS, RANA DATTA AND SUBHANGKAR NANDY ENHANCING THE BIOAVAILABILITY OF SIMVASTATIN USING MICROEMULSION DRUG DELIVERY SYSTEM	134-139
C SRINIVAS, S. VANITHA SAGAR OUANTIFICATION OF (-) EPICATECHIN BY HPTLC METHOD IN CASSIA FISTULA CRUDE DRUG, LAB EXTRACT AND	140-142
COMMERCIAL EXTRACT PRAVEEN PATIDAR, DARSHAN DUBEY AND KAMLESH DASHORA	
A CROSS SECTIONAL SURVEY ON KNOWLEDGE, ATTITUDE AND PRACTICE OF STAFF MEMBERS TOWARDS DAILY WATER INTAKE DURING WORKING HOURS IN INTERNATIONAL MEDICAL UNIVERSITY, MALAYSIA.	143-145
KINGSTON RAJIAH	
COMPARING THE FEFECT OF ANTIDIARETIC ACTIVITY OF ANDROGRAPHIS PANICULATA SALACIA RETICULATA AND	146.140
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N PREM KUMAR SOUMYA K VIJAYAN IN DHARSANA SEENA K XC ANJANA A KD	146-149
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA.K.VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UV-VIS SPECTROPHOTOMETRY	146-149 150-152
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA.K.VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UV-VIS SPECTROPHOTOMETRY ANEESH T.P, A.RAJASEKARAN	146-149 150-152
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA.K.VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UV-VIS SPECTROPHOTOMETRY ANEESH T.P, A.RAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF TABEBULA ROSEA AND SOLANUM PUBESCENS AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS	146-149 150-152 153-156
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA.K.VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UV-VIS SPECTROPHOTOMETRY ANEESH T.P, A.RAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBUIA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEMAMALINI. K, RAMYA KRISHNA. V, DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC	146-149 150-152 153-156 157-162
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA.K.VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UV-VIS SPECTROPHOTOMETRY ANEESH T.P, A.RAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBUIA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEMAMALINI. K, RAMYA KRISHNA. V, DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF <i>MUKIA MADERASPATANA</i> (L.) M.ROEM. (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL NADU, INDIA	146-149 150-152 153-156 157-162
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA.K.VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UV-VIS SPECTROPHOTOMETRY ANEESH T.P, A.RAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBUIA ROSEA</i> AND SOLANUM PUBESCENS AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEMAMALINI. K, RAMYA KRISHNA. V, DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF <i>MUKIA MADERASPATANA</i> (L.) M.ROEM. (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL NADU, INDIA MALLIKADEVI, T, S. PAULSAMY, S. JAMUNA AND K. KARTHIKA DESIGN, DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN	146-149 150-152 153-156 157-162 163-168
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA.K.VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UV-VIS SPECTROPHOTOMETRY ANEESH T.P, A.RAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF TABEBUIA ROSEA AND SOLANUM PUBESCENS AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEMAMALINI. K, RAMYA KRISHNA. V, DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF MUKIA MADERASPATANA (L.) M.ROEM. (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL NADU, INDIA MALLIKADEVI, T., S. PAULSAMY, S. JAMUNA AND K. KARTHIKA DESIGN, DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C. JAGDALE, SOMNATH A. PATIL, BHANUDAS S. KUCHEKAR HPTLC METHOD FOR QUANTITATIVE DETERMINATION OF GALLIC ACID IN ETHANOLIC ROOT EXTRACT OF <i>DIOSPYRUS</i>	146-149 150-152 153-156 157-162 163-168 169-174
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA.K.VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UV-VIS SPECTROPHOTOMETRY ANEESH T.P, A.RAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBUIA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEMAMALINI. K, RAMYA KRISHNA. V, DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF <i>MUKIA MADERASPATANA</i> (L.) M.ROEM. (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL NADU, INDIA MALLIKADEVI, T, S. PAULSAMY, S. JAMUNA AND K. KARTHIKA DESIGN, DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C. JAGDALE, SOMNATH A. PATIL, BHANUDAS S. KUCHEKAR HPTLC METHOD FOR QUANTITATIVE DETERMINATION OF GALLIC ACID IN ETHANOLIC ROOT EXTRACT OF <i>DIOSPYRUS</i> <i>FERREA</i> (WILLD.) BAKH AND AERVA LANATA (L.) JUSS. EX SCHULTES –A POTENT INDIAN MEDICINAL PLANTS. R.VIJAYALAKSHMI AND R.RAVINDHRAN	146-149 150-152 153-156 157-162 163-168 169-174
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA.K.VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UV-VIS SPECTROPHOTOMETRY ANEESH T.P, A.RAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBUIA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEMAMALINI. K, RAMYA KRISHNA. V, DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF <i>MUKIA MADERASPATANA</i> (L.) M.ROEM. (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL NADU, INDIA MALLIKADEVI, T., S. PAULSAMY, S. JAMUNA AND K. KARTHIKA DESIGN, DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C. JAGDALE, SOMNATH A. PATIL, BHANUDAS S. KUCHEKAR HPTLC METHOD FOR QUANTITATIVE DETERMINATION OF GALLIC ACID IN ETHANOLIC ROOT EXTRACT OF <i>DIOSPYRUS</i> <i>FERREA</i> (WILLD.) BAKH AND AERVA LANATA (L.) IUSS. EX SCHULTES – A POTENT INDIAN MEDICINAL PLANTS. R.VIJAYALAKSHMI AND R.RAVINDHRAN SCREENING OF <i>POLYCARPAEA CORYMBOSA</i> LAM. (CARYOPHYLACEAE) FOR ITS IN VITRO ANTIOXIDANT ACTIVITY SINDHU S AND S MANORAMA	146-149 150-152 153-156 157-162 163-168 169-174 175-178
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA.K.VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UV-VIS SPECTROPHOTOMETRY ANEESH T.P. A.RAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBULA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEMAMALINI. K, RAMYA KRISHNA. V, DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF <i>MUKIA MADERASPATANA</i> (L.) M.ROEM. (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL NADU, INDIA MALLIKADEVI, T. S. PAULSAMY, S. JAMUNA AND K. KARTHIKA DESIGN, DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C. JAGDALE, SOMNATH A. PATIL, BHANUDAS S. KUCHEKAR HPTLC METHOD FOR QUANTITATIVE DETERMINATION OF GALLIC ACID IN ETHANOLIC ROOT EXTRACT OF <i>DIOSPYRUS</i> <i>FERREA</i> (WILLD.) BAKH AND AERVA LANATA (L.) JUSS. EX SCHULTES –A POTENT INDIAN MEDICINAL PLANTS. RVIJAYALAKSHMI AND R.RAVINDHRAN SCREENING OF <i>POLYCARPAEA CORYMBOSA</i> LAM. (CARYOPHYLACEAE) FOR ITS IN VITRO ANTIOXIDANT ACTIVITY SINDHU S AND S MANORAMA PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF <i>SESBANIA SESBAN</i> (L.) MERR. T MYTHIU I AND R EAVINDHRAN	146-149 150-152 153-156 157-162 163-168 169-174 175-178 175-178
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA.K.VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UV-VIS SPECTROPHOTOMETRY ANEESH TP, A.RAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBUIA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEMAMALINI. K, RAMYA KRISHNA. V, DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF <i>MUKIA MADERASPATANA</i> (L.) M.ROEM. (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL NADU, INDIA MALLIKADEVI, T, S. PAULSAMY, S. JAMUNA AND K. KARTHIKA DESIGN, DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C. JAGDALE, SOMNATH A. PATIL, BHANUDAS S. KUCHEKAR HPTLC METHOD FOR QUANTITATIVE DETERMINATION OF GALLIC ACID IN ETHANOLIC ROOT EXTRACT OF <i>DIOSPYRUS</i> <i>FERREA</i> (WILLD.) BAKH AND AERVA LANATA (L.) IUSS. EX SCHULTES –A POTENT INDIAN MEDICINAL PLANTS. R.VIJAYALAKSHMI AND R.RAVINDHRAN SCREENING OF <i>POLYCARPAEA CORYMBOSA</i> LAM. (CARYOPHYLACEAE) FOR ITS IN VITRO ANTIOXIDANT ACTIVITY SINDHU S AND S MANORAMA PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF <i>SESBANIA SESBAN</i> (L.) MERR. T. MYTHILI AND R. RAVINDHRAN EVALUATION OF VARIOUS NATURAL SUSPENDING AGENTS FOR ITS SUSPENDING BEHAVIOUR USING PARACETAMOL AS MODEL DRUG FOR SUSPENDING MATURAL SUSPENDING AGENTS FOR ITS SUSPENDING BEHAVIOUR USING PARACETAMOL AS MODEL DRUG FOR NURSPENDING	146-149 150-152 153-156 157-162 163-168 169-174 175-178 179-182 183-186
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA.K.VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UVVIS SPECTROPHOTOMETRY ANEESH TP, A.RAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBUIA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEMAMALINI. K, RAMYA KRISHNA. V, DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF <i>MUKIA MADERASPATANA</i> (L.) M.ROEM. (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL NADU, INDIA MALLIKADEVI, T, S. PAULSAMY, S. JAMUNA AND K. KARTHIKA DESIGN, DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C. JAGDALE, SOMNATH A. PATIL, BHANUDAS S. KUCHEKAR HPTLC METHOD FOR QUANTITATIVE DETERMINATION OF GALLIC ACID IN ETHANOLIC ROOT EXTRACT OF <i>DIOSPYRUS</i> <i>ERREA</i> (WILLD.) BAKH AND AERVA LANATA (L.) IUSS. EX SCHULTES – A POTENT INDIAN MEDICINAL PLANTS. R.VIJAYALAKSHMI AND R.RAVINDHRAN SCREENING OF <i>POLYCARPAEA CORYMBOSA</i> LAM. (CARYOPHYLACEAE) FOR ITS IN VITRO ANTIOXIDANT ACTIVITY SINDHU S AND S MANORAMA PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF <i>SESBANIA SESBAN</i> (L.) MERR. T. MYTHILI AND R. RAVINDHRAN EVALUATION OF VARIOUS NATURAL SUSPENDING AGENTS FOR ITS SUSPENDING BEHAVIOUR USING PARACETAMOL AS MODEL DRUG FOR SUSPENSION NAGARGOJE SUDAM, BAWASKAR MANISH, MAHAJAN RITESH, PATIL SACHIN, M.PRATNAPARKHI, CHAUDHARI SHILPA	146-149 150-152 153-156 157-162 163-168 169-174 175-178 179-182 183-186
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYAK.VIJAYAN, J.N.DHARSANA, SEENA K.X.C, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UV-VIS SPECTROPHOTOMETRY ANEESH T.P. A.RAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBULA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEMAMALINI. K, RAMYA KRISHNA. V, DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF <i>MUKIA MADERASPATANA</i> (L.) M.ROEM. (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL NADU, INDIA MALLIKADEVI, T., S. PAULSAMY, S. JAMUNA AND K. KARTHIKA DESIGN, DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C. JAGDALE, SOMNATH A. PATIL, BHANUDAS S. KUCHEKAR HPTIC METHOD FOR QUANTITATIVE DETERMINATION OF GALLIC ACID IN ETHANOLIC ROOT EXTRACT OF <i>DIOSPYRUS</i> <i>FERREA</i> (WILLD.) BAKH AND AERVA LANATA (L.) IUSS. EX SCHULTES –A POTENT INDIAN MEDICINAL PLANTS. RVIJAYALAKSHMI AND R.RAVINDHRAN SCREENING OF <i>POLYCARPAEA CORYMBOSA</i> LAM. (CARYOPHYLACEAE) FOR ITS IN VITRO ANTIOXIDANT ACTIVITY SINDHU S AND S MANORAMA PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF <i>SESBANIA SESBAN</i> (L.) MERR. T. MYTHILI AND R. RAVINDHRAN EVALUATION OF VARIOUS NATURAL SUSPENDING AGENTS FOR ITS SUSPENDING BEHAVIOUR USING PARACETAMOL AS MODEL DRUG FOR SUSPENSION NAGARGOJE SUDAM, BAWASKAR MANISH, MAHAJAN RITESH, PATIL SACHIN, M.PRATNAPARKHI, CHAUDHARI SHILPA STUDY OF ANTI-INFLAMMATORY, ANALGESIC AND ANTIPYRETIC ACTIVITY OF NOVEL ISATIN DERIVATIVES VENKATESHWARLU E, VENKATESHWAR RAO J, UMASANKAR K, DHEERAJ G	146-149 150-152 153-156 157-162 163-168 169-174 175-178 175-178 179-182 183-186 183-186
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA K. VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UV-VIS SPECTROPHOTOMETRY ANEESH TP, A.RAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBULA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEMAMALINI, K, RAMYA KRISHNA, V, DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF MUKIA MADERASPATANA (L.) M.ROEM. (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL NADU, INDIA MALLIKADEVI, T, S. PAULSAMY, S. JAMUNA AND K. KARTHIKA DESIGN, DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C, JACDALE, SOMNATH A. PATIL, BHANUDAS S. KUCHEKAR HPTLC METHOD FOR QUANTITATIVE DETERMINATION OF GALLIC ACID IN ETHANOLIC. ROOT EXTRACT OF <i>DIOSPYRUS</i> <i>FERREA</i> (WILLD) BAKH AND AERVA LANATA (L.) JUSS. EX SCHUITES –A POTENT INDIAN MEDICINAL PLANTS, R.VIJAVALKSHMI AND R.RAVINDHRAN SCREENING OF <i>POLYCARPAEA CORYMBOSA</i> LAM. (CARYOPHYLACEAE) FOR ITS IN VITRO ANTIOXIDANT ACTIVITY SINDHU S AND S MANORAMA PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF <i>SESBANIA SESBAN</i> (L.) MERR. T. MYTHILI AND R. RAVINDHRAN EVALUATION OF VARIOUS NATURAL SUSPENDING AGENTS FOR ITS SUSPENDING BEHAVIOUR USING PARACETAMOL AS MOODEL DRUG FOR SUSPENSION NAGARGOJE SUDAM, BAWASKAR MANISH, MAHAJAN RITESH, PATIL SACHIN, M.P.RATNAPARKHI, CHAUDHARI SHILPA STUDY OF ANTI-INFLAMMATORY, ANALGESIC AND ANTIPYRETIC ACTIVITY OF NOVEL ISATIN DERIVATIVES VENKATESHWARLU E, VENKATESHWAR RAO J, UMASANKAR K, DHEERAJ G CHROMATOGRAPHIC FINGERPRINTING AND CLUSTERING OF <i>PLANTAGO MAJOR</i> L FROM DIFFERENT AREAS IN INDONESIA	146-149 150-152 153-156 157-162 163-168 169-174 175-178 175-178 179-182 183-186 183-186
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA K.VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UVVIS SPECTROPHOTOMETRY ANEESH TP, A.RAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBUJA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEMAMALINI. K, RAMYA RRISHNA. V, DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF <i>MUKIA MADERASPATANA</i> (L.) M.ROEM. (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL NADU, INDIA MALLIKADEVU, T., S. PAULSAMY, S. JAMUNA AND K. KARTHIKA DESIGN, DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C. JAGDALE, SOMNATH A. PATIL, BHANUDAS S. KUCHEKAR HPTIC METHOD FOR QUANTITATIVE DETERMINATION OF GALLIC ACID IN ETHANOLIC ROOT EXTRACT OF <i>DIOSPYRUS</i> <i>FERREA</i> (WILLD.) BAKH AND AERVA LANATA (L.) JUSS. EX SCHUITES – A POTENT INDIAN MEDICINAL PLANTS. RVIJAVALAKSIMI AND R.RAVINDHRAN SCREENING OF <i>POLYCARPAEA CORVMBOSA</i> LAM. (CARVOPHYLACEAE) FOR ITS IN VITRO ANTIOXIDANT ACTIVITY SINDHU S AND S MANORAMA PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF <i>SESBANIA SESBAN</i> (L.) MERR. T. MYTHILI AND R. RAVINDHRAN EVALUATION OF VARIOB AND ANTIMICROBIAL ACTIVITY OF SESBANIA SESBAN (L.) MERR. T. MYTHILI AND R. RAVINDHRAN EVALUATION OF VARIOB AND ANTIMICROBIAL ACTIVITY OF SESBANIA SESBAN (L.) MERR. T. MYTHILI AND R. RAVINDHRAN EVALUATION OF VARIOB AND ANTIMICROBIAL ACTIVITY OF NOVEL ISATIN DERIVATIVES VENKATESHWARLU E, VENKATESHWAR RAO J, UMASANKAR K, DHEERAJ G CHROMATOGRAPHIC FINGERPRINTING AND CLUSTERING OF <i>PLANTAGO MAJOR</i> L FROM DIFFERENT AREAS IN INDONESIA 32351111 AZMINAH	146-149 150-152 153-156 157-162 163-168 169-174 175-178 179-182 183-186 183-186 187-190 191-195
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA. SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA K. VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UVVIS SPECTROPHOTOMETRY ANEESH TP, A.RAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBUIA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEMAMALINI. K, RAMYA KRISHNA. V, DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF <i>MUKIA MADERASPATANA</i> (L.) MROEM. (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL NADU, INDIA MALLIKADEVI, T., S. PAULSAMY, S. JAMUNA AND K. KARTHIKA DESIGN, DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C. JAGDALE, SOMNATH A. PATIL, BHANUDAS S. KUCHEKAR HPTLC METHOD FOR QUANTITATIVE DETERMINIATION OF GALLIC ACID IN ETHANOLIC. ROOT EXTRACT OF <i>DIOSPYRUS</i> <i>FERREA</i> (WILLD, BAKH AND ARRA'LANATA (L.) IUSS. EX SCHULTES –A POTENT INDIAN MEDICINAL PLANTS. R.VIJAYALAKSHMI AND R.RAVINDHRAN SCREENING OF <i>POLYCARPAEA CORYMBOSA</i> LAM. (CARYOPHYLACEAE) FOR ITS IN VITRO ANTIOXIDANT ACTIVITY SINDHU S AND S MANORAMA PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF <i>SESEANIA SESBAN</i> (L.) MERR. T MYTHILI AND R. RAVINDHRAN EVALUATION OF VARIOUS NATURAL SUSPENDING AGENTS FOR ITS SUSPENDING BEHAVIOUR USING PARACETAMOL AS MODEL DRUG FOR SUSPENSION NAGARGOJE SUDAM, BAWASKAR MANISH, MAHAJAN RITESH, PATIL SACHIN, M.P.RATNAPARKHI, CHAUDHARI SHILPA STUDY OF ANTI-INFLAMMATORY, ANALGESIC AND ANTIPYRETIC ACTIVITY OF NOVEL ISATIN DERIVATIVES VENKATESHWARLU E, VENKATESHWAR RAO J, UMASANKAR K, DHEERAJ G CHROMATOGRAPHIC INGERPRINTING AND CLUSTERING OF <i>PLANTAGO MAIOR</i> L FROM DIFFERENT AREAS IN INDONESIA 'ZIGTITI, JAZMINAH SYNTHESIS AND BIOLOGICAL ACTIVITY OF PEPTIDE DERIVATIVES	146-149 150-152 153-156 157-162 163-168 169-174 175-178 175-178 179-182 183-186 183-186 187-190 191-195
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N. PREM KUMAR, SOUMYA.K.VIJAYAN, J.N.DRARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UV-VIS SPECTROPHOTOMETRY ANEESH TP, ARAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBULA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEMAMALINI, K, RAMYA KRISHNA, V, DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF <i>MURIA MADERASPATANA</i> (L_) M.ROEM, (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL NADU, INDIA MALLIKADEVI, T, S. PAULSAMY, S. JAMUNA AND K. KARTHIKA DESIGN, DEVLOPMENT AND EVALUATION OF FLOATINE TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C. JAGDALE, SOMNATH A. PATIL, BHANUDAS S. KUCHEKAR HPTLC METHOD FOR OUANTITATIVE DETERMINATION OF GALLIC ACID IN ETHANOLIC. ROOT EXTRACT OF <i>DIOSPYRUS</i> <i>FEREFA</i> (WILLD). BAKH AND AERVA LANATA (L_) IUSS. EX SCHUITES – A POTENT INDIAN MEDICINAL PLANTS. R.VIJAVALAKSHMI AND R.RAVINDHRAN SCREENING OF <i>POLYCARPAEA CORYMBOSA</i> LAM. (CARVOPHYLACEAE) FOR ITS IN VITRO ANTIOXIDANT ACTIVITY SINDHU S AND S MANORAMA PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF <i>SESBANIA SESBAN</i> (L_) MERR. T. MYTHILI AND R. RAVINDHRAN EVALUATION OF VARIOUS NATURAL SUSPENDING AGENTS FOR ITS SUSPENDING BEHAVIOUR USING PARACETAMOL AS MODEL DRUG FOR SUSPENSION NAGARGOJE SUDAM, BAWASKAR MANISH, MAHAJAN RITESH, PATIL SACHIN, M.P.RATNAPARKHI, CHAUDHARI SHILPA STUDY OF ANTI-INFLAMMATORY. ANALGESIC AND ANTIPYRETIC ACTIVITY OF NOVEL ISATIN DERIVATIVES VENKATESHWARLU E, VENKATESHWAR RAO J, UMASANKAR K, DHEERAJ G CHROMATOGRAPHIC FINGERPRINTING AND CLUSTERING OF <i>PLANTAGO MAJOR</i> L.FROM DIFFERENT AREAS IN INDONESIA MUNDONESIA MUNDONESIA MUNDINGSIA MUNDHANY, ANILGESIC AND ANTIPYRETIC ACTIV	146-149 150-152 153-156 157-162 163-168 169-174 175-178 175-178 179-182 183-186 183-186 187-190 191-195
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING. N. PREM KUMAR, SOUMYAK, VIJAYAN, IN. DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UVVIS SPECTROPHOTOMETRY ANEESH TP, ARAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBUIA ROSEA</i> AND SOLANUM PUBESCENS AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN KATS HEMAMALINI, K, RAMYA KRISHNA, V, DR, ANURAG BHARGAV AND DR, UMA VASIREDDY ANAUYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF <i>MURIA MADERASPATANA</i> (L.) M.ROEM. (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL KADU. INDIA MALLIKABEVY, S. JANUNA AND K. KARTHIKIA DESIGN, DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C. JAGDALE, SOMNATH A. PATIL, BHANUDAS S. KUCHEKAR HFILC METHOD FOR QUANTITATIVE DETERMINATION OF GALLIC ACID IN ETHANOLIC. ROOT EXTRACT OF <i>DIOSPYVUS</i> <i>FERREA</i> (WILLID) BAKH AND ARAVINDHRAN SCREENING OF <i>POLYCARPIAEA CORYMBOSA</i> LAM. (CARVOPHYLACEAE) FOR ITS IN VITRO ANTIOXIDANT ACTIVITY SINDHU S AND S MANORAMA PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF <i>SESBANIA SESBAN</i> (L.) MERR. T MYTHILI AND R. RAVINDHRAN EVALUATION OF VARIOUS NATURALS USPENDING AGENTS FOR ITS SUSPENDING BEHAVIOUR USING PARACETAMOL AS MODEL DRUG FOR SUSPENSION NACARGOG FOR SUSPENSION NACARGOR SUSPENSION NACARGOG FOR SUSPENSION NACARGOR SUSPENSION NACARGOG FOR SUSPENSION NACARGOR SUSPENSION NACARGO	146-149 150-152 153-156 157-162 163-168 169-174 175-178 179-182 183-186 183-186 187-190 191-195 196-200
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITRO SCREENING N PREM RUMAR, SOUMYA KUJAYAN, IA DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UVYIS SPECTROPHOTOMETRY ANEESH F2, ARAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF TABEBURA ROSEA AND SOLANUM PUBESCENS AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEMMALINI, K, RAMYA KRISINA, V, DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF MUKIA MADERASPATANA (L.) M.ROEM, (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTBEND ISTRICTS OF TAMIL, NADU, INDIA MALLIKADEVI, T, S. PAULSAMY, S. JAMUNA AND K. KARTHIKA DESIGN, DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN WATI C. JACADE, SOMNATH A PATTI, BHANDADS S. KUCHEKAR HPTLC METHOD FOR QUANTITATIVE DETERMINATION OF GALLIC ACID IN ETHANOLIC. ROOT EXTRACT OF <i>DIOSPIPUS</i> <i>ERREAL</i> (WILLD, BAKH AND AERVALANATA (L.) LUSS EX SCHULTES – A POTENTI INDIAN MEDICINAL PLANTS. RVIJAVALKSHMI AND R.RAVINDHRAN SCREENING OF POLICARPAEA CORYMBOSA LAM. (CARVOPHYLACEAE) FOR ITS IN VITRO ANTIOXIDANT ACTIVITY SINDHU S AND S MANORAMA PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF <i>SESDANIA SESDAN</i> (L.) MERR. T WITHILI AND R. RAVINDHRAN EVALUATION OF VARIOUS NATURAL SUSPENDING AGENTS FOR ITS SUSPENDING BEHAVIOUR USING PARACETAMOL AS MODEL DRUG FOR SUSPENSION NACARGOGIE SUDAM, BAWASKAR MANISH, MAHAJAN RITESH, PATIL SACHIN, M.PRATNAPARKHI, CHAUDHARI SHILPA STUDY OF ANTI-INFLAMMATORY, ANALGESIC AND ANTIPYRETIC ACTIVITY OF NOVEL ISATIN DERIVATIVES VENKATESHWARL U, VENKATESHWAR RAO, J. UMASANKAR K, DHEERAJ G CHROMATOGRAPHIC FINGERPRINTING AND CLUSTERING OF <i>PLANTAGO MAJOR</i> L. FROM DIFFERENT AREAS IN INDONESIA 3025111, AZMINAH SYNTHESIS AND BIOLOGICAL ACTIVITY OF PEPTIDE DERIVATIVES OF 2.HYDROXY.5-(6-1	146-149 150-152 153-156 157-162 163-168 169-174 175-178 179-182 183-186 187-190 191-195 196-200 201-206 207-210
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA. SALACIA RETICULATA AND OCHUMU SANCTUM PEY NUTHOR SCREENING N. PREM KUMAR, SOUMYA.K.VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING ULVIS SPECTROPHOTOMETRY ANEESH T.P. ARAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBUIA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTONICITY IN RATS HEPATOPROTECTIVE ACTIVITY OF <i>TABEBUIA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTONICITY IN RATS HEPATOPROTECTIVE ACTIVITY OF <i>TABEBUIA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTONICITY IN RATS HEMAMALINI, K., RAMTA KIRISINA, V. DR. ANURAG BHARGAV AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WIGOLE PLANT METHANOLIC EXTRACT OF <i>MUKAM MODERASPATANA</i> (L.). M.ROEM, (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL NADU, INDIA MALLIKADEVI, T., S. PAULSAMY, S. JAMUNA AND K. KARTHIKA DESIGN, DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C. JACOALE, SOMNATH A. PATIL, BHANUDAS S. KUCHEKAR HPTLC METHOD FOR QUANTITATIVE DETERMINATION OF GALLIC ACID IN ETHANOLIC. ROOT EXTRACT OF <i>DIOSPIRIS</i> <i>ERREA</i> (WILLD, JAKH AND ARVA JANATA (L.) JUSS. EX SCHUTES – A FOTENT INDIAN MEDICINAL PLANTS, R.VIJAVALAKSIMI AND RAVINDHRAN SCREENING OF <i>POLICARPIEA CORYMBOSA</i> LAM. (CARVOPHYLACEAE) FOR ITS IN VITRO ANTIOXIDANT ACTIVITY SINDHU S AND S MANORAMA PIYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF <i>SESBANIA SESBAN</i> (L.) MERR. T. MYTHILI AND R. RAVINDHRAN EVALUATION OF VARIOUS NATURAL SUSPENDING AGENTS FOR ITS SUSPENDING BEHAVIOUR USING PARACETAMOL AS MODEL DURG COS SUSPENSION NAGARGOJE SUDAM, BAWASKAR MANISH, MAHAJAN RITESH, PATIL SACHIN, M.PRATNAPARKHI, CHAUDHARI SHILPA STUDY OF ANTI-INFLAMMATORY. ANALGESIC AND ANTIPYRETIC ACTIVITY OF NOVEL ISATIN DERIVATI	146-149 150-152 153-156 157-162 163-168 169-174 175-178 175-178 179-182 183-186 187-190 191-195 196-200 201-206 207-210
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCINUM SAKUTUM PSI INVITUS CREEENING N. PREM KUMAR, SOUMYA K. VIJAVAN, J.N. DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS USING UV-VIS SPECTROPHOTOMETRY ANEESH TP, A.RAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBUIAR ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS HEPATOTOXICITY IN RATS HERATOTIXI PUBLICITY IN RATS IN HERATIL, BHANUDAS S. KUCHEKAR HIPTLC HERMOPTOR OUANTTATIVE DETERMINATION OF GALILC ACID IN FITHANOLIC ROT EXTRACT OF <i>DIOSPITUS</i> FERETA(UTUL) BUNAN AND REVA LINATA (L.) HUSS. EX SCHULTES -A POTENT INDIAN MEDICINAL PLANTS. RVIJAVALAKSHMI AND R.RAVINDHRAN SCREENING OF POULCARPAEL CORMARCA LANTICLES IN SCHULTES -A POTENT INDIAN MEDICINAL TOUTY SINDHU S AND SANAORAMA PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF SEESBAN/A SESBAN (L.) MERE, T. MYTTOHEMICAL SCREENING AND ANTIMICRO	146-149 150-152 153-156 157-162 163-168 169-174 175-178 175-178 179-182 183-186 183-186 183-180 201-200 201-200 201-200
COMPARING THE EFFECT OF ANTIDUABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA, SALACIA RETICULATA AND OCHUMIS ASACLUM BY INVITIOS CREEENING N. PREM KUMAR, SOUMYA K. VIJAYAN, J.N. DHARSANA, SEENA K.XC, ANJANA A.KD METHOD DEVELOPMENT AND YALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMATI AND YALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMATIN SING U.V.YS. SPECTROPIOTOMETRY ANEESIN TP, A RAJASEKARAN HEPATOTOXICTIVI IN RATS HEPATOTOXICTIVI IN RATS HEMAMALINI, K, RAMYA KRISINA, V, DR. ANURAG BHARGAY AND DR. UMA VASIREDDY ANALYSIS FOR PHYTOCEUTICALS AND BIOINFORMATICS APPROACH FOR THE EVALUATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF MUGIA MADEASPATANA (L.) M.ROFM, CUCURBITACEAE]. – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMIL NADU. INDIA MALLIKADEVI, S. PAULSANY, S. JARUNA AND K. KARTHIKA DESIGN, DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C, JACDALE, SOMNATH A. PATIL, BHANUDAS S. KUCHEKAR HPTLC METHOD FOR QUANTITIVE DETERMINITON OF GALILC ACID IN FTHANOLIC ROOT EXTRACT OF DIOSPIRUS <i>ERREA</i> (WILLD) BAKH AND AREVA LAWATA (L.) JUSS. EX SCHUITES – A POTENT INDIAN MEDICINAL PLANTS. RVIJAYALAKSIMI AND RAWINDHAN SCREENING OF POLUCAPIZE COMPHOSA LAM. (CANYOPHYLACEAE) FOR ITS IN VITRO ANTIOXIDANT ACTIVITY SINDHO S AND S MANORAMA PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF SESBANA SESBAN (L.) MERR. T WITHILI AND R. RAVINDHRAN EVALUATION OF VAHIOUS NATURAL SUSPENDING AGENTS FOR ITS SUSPENDING BEHAVIOUR USING PARACETAMOL AS MODEL DRUG FOR SUSPENSION NAGARCOJE SUDAM, BAWASAKAR MANISH, MAHAJAN RITESH, PATIL SACHIN, M. PRATNAPARKHI, CHAUDHARI SHILPA STUDY OF ANTI-INFLAMMATORY, ANALGESIC AND ANTIPYRETIC ACTIVITY OF NOVEL ISATIN DERIVATIVES VENKATESHWARLU E, VENKATESHWAR RAO, J. UMASANKAR K, DHEERAJ G CIRMONATOGRAPHIC FINGERPRINTING AND CLUSTERING OF PLANTAGO MAJOR L FROM DIFFERENT AREAS IN INDONESIA ZISTISTI, ALVINAMA UNANA SINGL, RAMESH KUMAR GUPTA DISERVATION OF ESTIMATE	146-149 150-152 153-156 157-162 163-168 169-174 175-178 175-178 179-182 183-186 183-186 187-190 201-206 201-206 207-210 211-214 215-224
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA. SALACIA RETICULATA AND OCIMUM SANCTUM BY INVITOS CREVENING N. PREM KUMAR, SOUMYA K VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHIOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORVAS USING ULYUS SPECTROPHOTOMETRY ANEESEN TP, ARAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABBEBUA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOPROTECTIVE ACTIVITY OF <i>TABBEBUA ROSEA</i> AND <i>SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOPROTECTIVE ACTIVITY OF <i>TABBEBUA</i> ROSEA AND SOLANUM PUBESCENS AGAINST PARACETAMOL INDUCED HEPATOPROTECTIVE ACTIVITY OF <i>TABBEBUA</i> ROSEA AND SOLANUM PUBESCENS AGAINST PARACETAMOL INDUCED HEPATOPROTECTIVE AND FYANDAMICS APPEOACH FOR THE FUNDATION OF THERAPETIC PROPERTIES OF WHOLE PLANT METHANOLIC EXTRACT OF <i>MURIA MADERASPITANA</i> (L.) M ROEM, (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN VESTERR DISTRICTS OF TABILTS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C, JAGDALE, SOMNATH A, PATIL, BHANUDAS S, KUCHEKAR HITL CMETHOP FOR QUANTITATUR DE ETERMINATION OF GALLIC ACID IN ETHANOLIC ROOT EXTRACT OF <i>DIOSPIRUS</i> <i>ERRENI</i> , OF POLIVARTIATUR DE ETERMINATION OF GALLIC ACID IN ETHANOLIC ROOT EXTRACT OF <i>DIOSPIRUS</i> <i>ERRENI</i> , QUILLD BAKH AND ARAVINDHRAN SCREENING OF POLIVARATIATUR DE TERMINATION OF GALLIC ACID IN ETHANOLIC ROOT EXTRACT OF <i>DIOSPIRUS</i> <i>ERRENI</i> , QUILLD BAKH AND ARAVINDHRAN SCREENING OF POLIVARATIATUR DE TERMINATION OF SESSANIA SESBAY (L.) MERR. TUYHTULI AND R. RAVINDHRAN EVALUATION OF VARIOUS NATURAL SUSPENDING AGENTS FOR ITS SUSPENDING BEHAVIOUR USING PARACETAMOL AS MODEL DEUC POR SUSPENSION NAGARGOJE SUDAM, BAWASKAR MANISH, MAHAJAN RITESH, PATIL SACHIN, M.P.RATNAPARKHI, CHAUDHARI SHILPA STUDY OF ANTI-UNFLAMMATOK, ANALGSIC AND ANTIPYRTIC ACTIVITY OF NOVEL ISATIN DERIVATIVES VENKTESTEMANG US, SUSUEL AND ANTIPHICROBIAL ACTIVITY OF NOVEL ISATIN DERIVATIVES VENKTESTEMA DE OF SUSPENSION NAGARGOJE SUDAM, BAWASKAR MANISH, MAHAJAN RITESH, PATHORYS 5	146-149 150-152 153-156 157-162 163-168 169-174 175-178 175-178 179-182 183-186 183-186 183-186 201-200 201-200 201-200
COMPARING THE EFFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA. SALACIA RETICULATA AND OCIMUM SANCTIM BY INVITED SCREENING N. PREM KUMAR, SOUMYA K VIJAYAN, J.N.DHARSANA, SEENA K.XC, ANJANA A.KD METHIOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORVS USING UVYS SPECTROPHOTOMETRY ANEESIN TP, ARAJASEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>TABEBULA ROSEA</i> AND SOLANUM PURESCENS AGAINST PARACETAMOL INDUCED HEPATOPROTECTIVE ACTIVITY OF <i>TABEBULA ROSEA</i> AND SOLANUM PURESCENS AGAINST PARACETAMOL INDUCED HEPATOPROTECTIVE ACTIVITY OF TABEBULA ROSEA AND SOLANUM PURESCENS AGAINST PARACETAMOL INDUCED HEPATOPROTECTIVE ACTIVITY OF TABEBULA ROSEA AND SOLANUM PURESCENS AGAINST PARACETAMOL INDUCED HEPATOPROTECTIVE ACTIVITY OF TABEBULAR SOLA ON DR. UMA VASIREDDY ANALYSIS FOR HENTOCEUTICASIA ND BIONFORMATICS APPROACH FOR THE FLAVILIATION OF THERAPETIC PROTERTIES OF WHOLE FLANT METHANOLIC EXTRACT OF <i>HUKIM MADERASPITANA</i> (L.) M ROEM, (CUCURBITACEAE) – A TRADITIONAL MEDICINAL PLANT IN WESTERN DISTRICTS OF TAMENTADOL HYDROCHLORIDE USING CHITOSAN SWATI C, JAGDALE, SOMNATH A PATIL, BHANUDAS S. KUCHEKAR HITLC METHOD FOR OLIVANTIATIVE DETERMINATION OF GALILC ACID IN ETHANOLIC ROOT EXTRACT OF <i>DIOSPIRUS ERREA</i> (MULLD) BARH AND AERVA LANATA (L.) INSS. EX SCHULTES –A POTENT INDIAN MEDICINAL PLANTS. R.VIJVALAKSINI AND RARVINDHRAN SCREEENING OF <i>POLICARPIAEA CORVINDOSA</i> LAM. (CARYOPHYLACEAE) FOR ITS IN VITRO ANTIOXIDANT ACTIVITY SINDHU S AND S MANORAMA PHYTOCHENICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF SESRANIA SESBAN (L.) MERR. T MYTHILI AND R. RAVINDHRAN SCRUEENING OF ASUSPENSION MAGARGOJE SUDAM, BAWASKAR MANISH, MARIJAN RITESH, PATL SACHIN, M.P.RATNAPARKHI, CHAUDHARI SHILPA STUDYO OF ANTI-MERAMATORY. ANALGESIC AND ANTIPYRETIC ACTIVITY OF NOVEL ISSIN PERATORING INSIGN PARACETAMOL AS MODEL DUCE OF SUSPENSION MAGARGOJE SUDAM, BAWASKAR MANISH, MARIJAN RITESH, PATL SACHIN, M.P.RATNAPARKHI, CAUDHARI SHILPA STUDYO OF ANTI-MERAMATORY. ANALGESIC AND ANTIPYRETIC ACTIVITY OF NOVEL ISSIN PARACE	146-149 150-152 153-156 157-162 163-168 169-174 175-178 175-178 179-182 183-186 183-186 183-186 191-195 201-200 201-200 201-200
COMPARING THE FFECT OF ANTIDIABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA. SALACIA RETICULATA AND OCIMUM SANCTURY BY INVITOS OSCREENING. N. PEEN KUMAR, SOUMYAK VUJAYAN, JA DHANSANA, SEENA KAY, ANJANA A KO METHOD DEVELOPMENT AND YALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOGAGE FORMS USING UNYS DESTEROPHOTOMENTRY. ANTESIS USING UNYS DESTENDED TO THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOGAGE FORMS USING UNYS DESTENDED TO THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOGAGE FORMS USING UNYS DESTENDED TO THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL THEATOPROFECTIVE ACTIVITY OF TABERUA BOSEA AND SOLANUM PUBESCENS AGAINST PARACETAMOL INDUCED HEPATOPROFECTIVE ACTIVITY OF TABERUA BOSEA AND SOLANUM PUBESCENS AGAINST PARACETAMOL INDUCED HEPATOPROFECTIVE ACTIVITY OF TABERUA BOSEA AND SOLANUM PUBESCENS AGAINST PARACETAMOL INDUCED HEPATOPROFECTIVE ACTIVITY OF TABERUA BOSEA AND SOLANUM PUBESCENS AGAINST PARACETAMOL INDUCED HEPATOPROFECTIVE ACTIVITY OF TABERUA BOSEA AND NE KARTHIKA DESIGN, DEVISOPHENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHLORIDE USING CHITOSAN SWATT CIAGDALE, SONNATH A PATU, BIAAUDAS S KUCHEKAR HEPTLC HEPHOD FOR OUANTTATIVE DETERNINATION OF GALLIC ACID IN ETHANOLIC ROOT EXTRACT OF <i>DIOSPRUS</i> FRERA (WILLO) BARIA AND ARKAN ANARYA ALANTA (L.) IUSS EX SCRUITES – A POTENT INDIAN MEDICINAL PLANTS. RVIJAZIAKSINMI AND R.RAVINDHRAN SCREENING OF POLICARREAG CORMOBOSI LAM, CCANOPHYLACEAEJ FOR ITS IN VITRO ANTIOXIDANT ACTIVITY SINDHU S AND S ANA ORAMA PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF SESBANIA SESBAN (L.) MERR. T MYTHILI AND R. RAVINDHRAN EXALUSTING OF VALOUS NATURAL SUSPENDING AGENTS FOR ITS SUSPENDING BEHAVIOUR USING PARACETAMOL AS MODEL DOUG OF SUSPENSION NAGARCOGE SUMARAMAN, BAWASKAR NANISH, MAHAJAN RITESH, PATIL SACHIN, MERATNAPARKHI, CHAUDHARI SHILPA STUDY OF ANTI-INFLAMMATORY, ANALGESIC AND ANTIPICRETIC ACTIVITY OF NOVEL ISATIN DERIVATIVES VENATESH KARUU E, VENATESHSKAN NANISH, MAHAJAN RITESH, PATIL SACHIN, MERATNAPARKHII	146-149 150-152 153-156 157-162 163-168 169-174 175-178 179-182 183-186 183-186 187-190 191-195 196-200 201-206 207-210 201-206 207-210
COMPARING THE FFECT OF ANTIDABETIC ACTIVITY OF ANDROGRAPHIS PANICULATA. SALACIA RETICULATA AND OCMUM SANCTUM BY INVITOS CRETENING N. PEEN KUMAR. SOUMYAK VUAYAN, JAX DHARSANA, SEENA KAY, ANJANA A KO METHOD DEVELOPHENT AND VALIDATION FOR THE ESTIMATION OF SILDOSIN IN BULK AND PHARMACEUTICAL DOSAGE FORMS SUNG UVYS SPECTROPHOTOMETRY ANEESI TP, ARAJSEKARAN HEPATOPROTECTIVE ACTIVITY OF <i>CREEDING ROSEA AND SOLANUM PUBESCENS</i> AGAINST PARACETAMOL INDUCED HEPATOPROTECTIVE ACTIVITY OF <i>CREEDING</i> ROSEA AND SOLANUM PUBESCENS AGAINST PARACETAMOL INDUCED HEPATOPROTECTIVE ACTIVITY OF <i>CREEDING</i> ROSEA AND SOLANUM PUBESCENS AGAINST PARACETAMOL INDUCED HEPATOPROTECTIVE ACTIVITY OF <i>CREEDING</i> ROSEA AND SOLANUM PUBESCENS AGAINST PARACETAMOL INDUCED HEPATOPROTECTIVE ACTIVITY OF CREEDING AND CARACETAMOL THE EVALUATION OF THERAPETIC PROFERITIS OF WHOLF PLANT METHANOLIC EXTRACT OF <i>VURCA MADERASPRANA</i> (L.) MADERA, (CUCURBITACEAE) – A TABUTIONAL MUDICINAL PLANT IN WESTERN DISTICTS OF TANIL NADU. INDIA MALIKADEVI, T. S. PAULSANY, S. JAMUNA AND K. KARTHIKA DESIGN. DEVELOPMENT AND EVALUATION OF FLOATING TABLETS OF TAPENTADOL HYDROCHICARLDE USING CHITOSAN SWATC I, GAOLAE, SONNATA A PATIL, BHANUDAS S. KUCHEAR HETLG, METHOD FOR QUANTITATIVE DETERMINATION OF GALLIC ACID IN ETHANOLIC ROOT EXTRACT O <i>P DIOSPHYLIS</i> FREES (WILLD) BARK AND ARENA LANATA (L.) INSISTEN DISTING STANDAUT ACTIVITY SINDHU S AN S MANORAMA PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF SESBANIA SESBAN (L.) MERR. T WYTHILI AND R. RAVINDHRAN EXTREMENG OF POLICARPARAEL GON/MOSAI LAM, (CARIOPHYLACEAE) FOR ITS IN VITRO ANTOXIADAT ACTIVITY SUNDHU S NANORAMA PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF SESBANIA SESBAN (L.) MERR. T WYTHILI AND R. RAVINDHRAN EXTREMENG OF ANUNDRAN SCREENING OF ANDROMA, BAWASKAR NANISI, MAHAJAN RITESH, PATIL SACHIN, M. PRATNAPARKHI, CHAUDHARI SHILPA STUDY OF ANTI-HERAPHINING AND ANTIMICROBIAL ACTIVITY OF SOLANDA SYS-(6-1000 -2.//ETHINI- 4.0200UHAZOUN, SUGHAPATANAN AN ANTIMICROBIAL ACTIVITY OF SOLANDA SYS-(6-1000 -2.//ETHINI- 4.0200UHAZO	146-149 150-152 153-156 157-162 163-168 169-174 175-178 179-182 183-186 183-186 191-195 196-200 201-206 201-206 207-210 211-214 225-231 235-238 239-243

Case Study

CIPROFLOXACIN INDUCED DRUG REACTION- A CASE REPORT

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248

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Research Article

CHROMATOGRAPHIC FINGERPRINTING AND CLUSTERING OF *PLANTAGO MAJOR* L. FROM DIFFERENT AREAS IN INDONESIA

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ABSTRACT

Plantago major L. is a ubiquitous herbaceous plant with many medicinal activities which has been extensively used in Indonesian traditional medicine. This plant grows at wide range of environment, has several subspecies and varieties, but could not be distinguished morphologically. It is important to develop an effective method for identification and quality assurance of *P. major*, thus the final product has reproducible quality. In this research, a chromatographic fingerprint method was developed for exploring and establishing the variation of chemical substances among different samples of *P. major* collected from 15 areas in Indonesia. The LC (liquid chromatography) data showed considerable variation of chemicals among *P. major* samples. Three chemo-types were visually developed from the LC profiles. The hierarchical clustering analysis also concluded that the samples were divided into three major clusters. Furthermore, the bio-active marker aucubin in this herb was quantitatively determined by a validated LC analysis. Chemo-type II and III were identified as "compound 1"-rich and aucubin-relatively rich chemo-types. These conclusions provide an important basis to establish good agriculture practice and select geo-authentic crude drug for *P. major* in Indonesia. The validated method was concluded to be suitable for fingerprint analysis for the quality control of *P. major*.

Keywords: Plantago major, Chromatographic fingerprinting, Clustering analysis, Aucubin, Column liquid chromatography

INTRODUCTION

Development of herbal-based drugs meet several obstacles, one of them is how to ensure the consistency or uniformity of their chemical contents. The chemical constituents of the herbal medicine products may vary depending on harvest seasons and time, plant origins, drying processes and other factors. Medicinal plants collected from different locations and environment may vary in types and levels of chemical components. Therefore, affect their efficacy.

P. major has several subspecies and varieties which could not be distinguished morphologically because of many intermediate forms. In Indonesia, *P. major* grows at a very wide range of regions, from 0 up to 3,300 m above sea level. However, most of them grow at 700 m above sea level or more^{1,2}. Their habitats include grasslands, agricultural areas, sides of roads and river side, forests and others, mainly on open fertile and rather hard land^{2,3}.

P. major contains various chemical compounds, one of them is iridoid glycosides⁴. Previous chemo-taxonomic study concluded that aucubin (Fig. 1) is a typical iridoid compound found in all genus of Plantago, including *P. major*⁵⁻⁹. Aucubin has shown wide pharmacological properties, as: hepatoprotective, anti-toxic, anti-inflammatory, antioxidant, anti-aging, anti-osteoporosis, neurotrophic, removal uric acid and diuretic¹⁰⁻¹⁵.



Fig. 1:Chemical structure of aucubin

Adequate identification and quality assurance of crude drug or extract are very important requirement in ensuring the reproducibility of drug. Therefore, support its safety and efficacy. Apart from the extraction process, the quality of extract is determined by the quality of crude drug used. In addition to subspecies and varieties of plants, plant constituents are also influenced by some environmental factors namely: soil (pH, nutrient content and water), climate (temperature, humidity, rainfall, wind speed) and biotic factors (competition with other plants, herbivores and parasites). Previous studies showed that chromatographic fingerprinting analysis is rapid, efficient, and appropriate method for authentication and quality assurance of herbal medicines. This method has been adopted by France, Germany, Britain, India, Japan, WHO, and Chinese SFDA to evaluate the quality of medicinal plants, such as *Centella asiatica, Salvia miltiorrhiza, Crocus sativus*, and *Flemingia philippinensis*¹⁶⁻²⁰.

In this paper, for the first time an LC method was developed for fingerprinting of *P. major* collected from different locations in Indonesia. In addition, the concentration of aucubin in 15 samples of this plant were compared.

MATERIALS AND METHODS

Plant Materials, Chemicals and Solvents

P. major were collected from 15 different regions in Indonesia (Table 1). Plants were uprooted and washed with tap water. Next, the flowers, petioles and roots were separated from the leaves. A whole plant was left for species determination. Voucher specimens of all the samples, morphologically authenticated by Professor Sutarjadi, were deposited at Center of Information and Development of Herbal Medicine, The University of Surabaya, Indonesia. Then, all of samples were dried (under indirect sunlight) and powdered (Mesh 20) before being stored at desiccator for use.

Aucubin (purity > 98%) was purchased from Fluka (Germany), water was prepared by degassing of water for injection (Ikapharmindo Putramas, Indonesia), acetonitrile and methanol were purchased from Mallinckrodt (USA).

Preparation of Plant Extract

Twenty five mg powder of each sample was extracted twice with 2 ml methanol under ultrasonic for 5 min, respectively. The extracts were adjusted to 5.0 ml with methanol. Finally, the solutions were filtered through a filter membrane (0.45 μm , Whatman) prior to HPLC analysis.

Instrumentation and Chromatographic Conditions

Chromatographic analysis was conducted with a Waters 1525 Binary HPLC Pump including 2 pumps and uv-vis detector. Separation was achieved on chromatography using a eurospher 100-5 C-18, with guard 250 mm x 4.6 mm ID (Knauer GmbH-Germany). The mobile phase consisted of acetonitrile and water (7:93). The flow rate was kept constant at 0.6 mL min⁻¹ and the run time was 10 min. The detector wavelength was set at 204 nm and the injection volume was 20 μ l.

Location	Population Code	Location (Lati	tude, Longitude)	Time of Collection
Kediri	KDR	7º48'36"S,	112º00'36"E	August, 2011
Madiun	MDN	7º37'30"S	111º30'54"E	July, 2011
Surabaya	SBY	7º17'24"S	112º43'36.48"E	August, 2011
Mojokerto	MJK	7º40'12"S	112º36'36"E	August 2011
Malang	MLG	7∘59'6"S	112º38'6"E	July, 2011
Salatiga	SLTG	7º20'24"S	110º29'42"E	July, 2011
Tegal	TGL	6º20'24"S	109º08'6"E	July, 2011
Purwokerto	PWK	7º26'2.4"S	109º14'57.12"E	July, 2011
Banyumas	BYM	7∘31'48"S	109º18'18"E	July, 2011
Cilacap	CLC	7º43'48"S	108º59'24"E	July, 2011
Cakranegara	CKR	8º35'42"S	116º08'42"E	July, 2011
Suranadi	SRN	8º34'S	116º06'E	February, 2012
Bonjeruk	BJR	8º39'28.8"S	116º13'33.6"E	February, 2012
Telaga Waru	TGW	8º37'19.2"S	116°07'4.8"E	February, 2012
Praya	PRY	8°42'S	116°14'E	February, 2012

Table 1:Geographical locations of P. major collected from different locations in Indonesia

Precision, Repeatability and Stability

The precision was determined by replicating injection of the same sample (sample KDR) solution for five times. The repeatability test was analyzed by injecting five independently prepared samples (sample KDR). The stability test was determined by four injections with one sample (sample TGW) solution during 24 h. The RSD of relative retention times and relative peak areas of each test were calculated.

Calibration, Linearity, Recovery

Standard stock solution of aucubin was prepared by dissolving certain amount of aucubin in 10 mL methanol to obtain a concentration of 210 mg L⁻¹. The concentrations of aucubin reference standard used for calibration were 2.1, 5.25, 10.5, 26.25, 52.5, 63, 105, 157.5 and 210 mg L⁻¹. Three injections were performed for each dilution. The standard curve was calibrated using the linear regression equation derived from the peak areas.

Recovery was assessed by the method of standard additions. Sample (20, 25, and 30 mg) were spiked by addition of 1.5 ml of stock solution, then extracted, processed, and quantified as described above. Experiment was repeated for three times. Recovery was calculated by comparing the concentration of aucubin resulted from measurement to the actual concentration.

Quantification of Aucubin

The concentrations of aucubin in different samples were calculated according to the regression parameters derived from the standard curve. Each sample was prepared in three replications and each replication was injected in three times.

Data Analysis

Four characteristic peaks in the chromatograms were selected and the peak of aucubin at retention time 4.9 min was used as a reference. Relative retention time (RRT, the ratio between retention time of characteristic peaks to that of reference peak) and relative peak area (RPA, the ratio between peak area of characteristic peaks to that of reference peak) of each characteristic peaks to reference were calculated in the chromatograms. The hierarchical clustering analysis (HCA) of 15 samples was performed based on the variation patterns of four chemical constituents of each sample using SPSS 17.0 software (SPSS Inc., USA).

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

In addition to iridoid glycosides, *P. major* also contains various chemical compounds classified to the group of flavonoids, terpenoids, alkaloids, cafeic acid derivatives, polysaccharides, fats, vitamins and organic acids⁴. Among others iridoid glycosides, aucubin seemed to be the most important iridoid glycosides and

could be used as a marker in chemo-taxonomic study of *P. major* species⁵⁻⁹.

Previous study showed that HPLC and HPTLC were mainly used for determining the aucubin contents in Plantago species^{9,21,22}. In this paper, an HPLC metabolite fingerprinting method was used and developed for quality control of *P. major*. Reversed phase liquid chromatography with C18 column and acetonitrile-water mobile phases were chosen to separate *P. major* substances. The optimized mobile phases systems are shown in Table 2. The acetonitrile-water (7:93) system was the final choice. Iridoid glycosides including aucubin are polar compounds and they have good solubility in polar system, hence solution rich in water had better resolution and could improve the peak form of aucubin.

Table 2. The mobile phases were used in optimization

Mobile Phases	Elution Mode
Acetonitrile-methanol (1:99)	Isocratic
Acetonitrile-methanol (2:98)	Isocratic
Acetonitrile-methanol (3:97)	Isocratic
Acetonitrile-methanol (5:95)	Isocratic
Acetonitrile-methanol (10:90)	Isocratic
Acetonitrile-methanol (20:80)	Isocratic
Acetonitrile-methanol (30:70)	Isocratic
Acetonitrile-water (5:95, 20:80, 90:10, 5:95)	Gradient
Acetonitrile-water (7:93)	Isocratic

To gain optimal extract, a preliminary study chose methanol as extraction solvent compared to n-hexane and acetone. In addition, extraction method (ultrasonic) and extraction time $(2 \times 5 \text{ min})$ was also selected. In the present study, the maximum absorbance of aucubin was observed on the uv-vis spectrophotometer. The detector wavelength of 204 nm was selected, thus more detectable peaks could be observed and the baseline was well improved around 204 nm on the chromatographic profiles.

Method Validation

For chromatographic fingerprinting, we selected aucubin as target compound. Aucubin showed many pharmacological properties¹⁰⁻¹⁵ and easily analyzed^{9,21,22}. Therefore, it was predicted as a good analytical marker as well as bio-active marker. Prior to determination of aucubin concentration and chromatographic fingerprinting of *P. major*, the method was validated. The validation parameters consisted of precision, repeatability, stability, calibration, linearity and recovery.

Precision, Repeatability and Stability Test

Precision testing was analyzed by replicate injection of the same sample (sample KDR) solution for five times consecutively in a day. The results are shown in Table 3. The RSD of RRT and RPA did not exceed 0.64 and 6.34%, respectively, which is indicative of the good reproducibility.

Peak No	Relative Retention Time Mean (RSD%)		R	elative Peak A Mean (RSD%)	rea)	
	Rpt	Rrt	Rst	Rpt	Rrt	Rst
1	0.74 (0.23)	0.73 (1.29)	0.71 (1.38)	2.13 (4.84)	2.53 (11.42)	1.44 (2.95)
2 (r)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)
3	1.45 (0.39)	1.45 (9.21)	1.46 (0.56)	0.11 (6.34)	0.10 (4.13)	0.061 (2.84)
4	1.84 (0.64)	1.84 (0.43)	1.87 (0.40)	0.06 (5.78)	0.09 (9.24)	0.37 (4.50)

Table 3:Results of relative retention times and relative peak areas of precision test, repeatability test and stability test of *P. major*populations from 15 locations in Indonesia

Rpt, Rrt, and Rst represent the results of relative retention times and relative peak areas of precision test, repeatability test, and stability test on LC fingerprint of *P. major* populations from different locations in Indonesia, respectively

The repeatability was evaluated by analyzing five independently prepared samples (sample KDR, 25 mg) of *P. major*. The results are shown in Table 3. The RSD of RRT and RPA were not more than 9.21 and 11.42%, respectively. These results suggested the method was feasible and rational for four analytes.

The stability of sample was determined by analyzing the same sample (sample TGW) every 6 h on a single day. During this period, the solution was stored in refrigerator. The results are shown in Table 3. The RSD of RRT and RPA were less than 1.38 and 4.50%, respectively. The similarity of these results indicated that the samples were not degraded during this period.

Calibration, Linearity and Recovery

Calibration curve of aucubin was designed from nine different concentrations (x) of aucubin against its peak area (y). Aucubin was accurately measured and dissolved in methanol to create stock solution. This solution then diluted to nine concentrations of aucubin. Calibration curve was conducted with linear regression analysis (Table 4). Plotting for aucubin showed good linearity ($r^2 >$



0.999) within the test ranges investigated. The limit of detection (LOD = 8.3911 mg L^{-1}) and the limit of quantification (LOQ = $27.9703 \text{ mg L}^{-1}$) were evaluated on the basis of signal-to-noise ratios of 3 and 10, respectively. Moreover, the recovery of aucubin (three independently prepared samples, sample CLC) was 104% and the RSD was 2.88%.

Quantitative Analysis and Chromatographic Fingerprint

The chromatographic fingerprints of *P. major* from different populations showed variation of chemical compounds which were especially in quantification (Fig. 2). One peak was identified by using external standard. In this study, according to the contents and biological activities of major constituents in *P. major*, the peak of aucubin was selected as reference peak. The level of aucubin in different samples varied significantly, from 0.44 to 1.72%, as listed in Table 4. LC fingerprint of *P. major* showed 3-4 peaks which could be selected as marker peaks in the fingerprint. These peaks can serve as characteristic peaks for identification of "unknown" samples and can distinguish three chemo-types based on chromatogram profiles.



Fig. 2:Original liquid chromatography profiles of methanol extracts, showing three chemotypes identified based on 15 *P. major* samples representing different populations (Peak 2 = aucubin)

Table 4:Contents of aucubin in different P. major populations

Region	Population Code	Regression equation ^a	Content (%) ^b
Kediri	KDR	y = 15431.2712 x + 692001.3808	0.70 (8.57)
Madiun	MDN	$r^2 = 0.9994$	1.16 (1.31)
Surabaya	SBY		1.10 (6.36)
Mojokerto	MJK		1.67 (4.50)
Malang	MLG		0.77 (9.37)
Salatiga	SLTG		1.54 (1.98)
Tegal	TGL		0.59 (9.73)
Purwokerto	PWK		0.61 (8.98)
Banyumas	BYM		0.71 (14.5)
Cilacap	CLC		1.63 (8.31)
Cakranegara	CKR		0.87 (7.68)
Suranadi	SRN		0.44 (5.68)
Bonjeruk	BJR		1.72 (1.21)
Telaga Waru	TGW		0.75 (8.53)
Prava	PRY		1.01 (14.38)

^a In the regression equation y = bx + a, y refers to the peak area, x the concentration of aucubin (mg L⁻¹), and r² is the correlation coefficient of the equation

^b Measured from 3 dry individuals of *P. major*, members in parenthesis indicate the RSD

The results of hierarchical clustering analysis showed that the samples from various regions could be divided into three groups (Fig. 3). It is important to be noted that the grouping of 15 *P. major* populations was in a good agreement with the visual comparison of their chromatograms, as presented by the chemo-types (Fig. 2). This clustering based on all peaks, not only based on aucubin content. Hence, concentration of aucubin varies in the same group (cluster I). Chemo-type I was identified as "compound 1"-low chemotype. Some of them rich in aucubin content. This chemo-type included all of

population except CKR and MDN. Furthermore, chemo-type II (sample CKR) and III (sample MDN) were suggested as "compound 1"-rich and aucubin-relatively rich chemo-types. This indicates that the place of origin significantly influences the content of constituents. This could certainly lead to variation of pharmacological effects. From this finding we could not justified that quality of *P. major* is only influenced by the source of plant since sub species, varieties, and cultivation practices also have possibilities in affecting the quality of plant.



Fig. 3:Hierarchical clustering analysis of 15 *P. major* samples (dendrogram using average linkage between groups) rescaled distance cluster combine

CONCLUSIONS

The bio-active constituent aucubin in *P. major* was quantitatively determined by a validated reverse-phase HPLC analysis. Three chemo-types of *P. major* were visually developed from the chromatographic profiles. The hierarchical clustering analysis further suggested that the samples were divided into three major groups. Chemo-types II and III were suggested as "compound 1"-rich and aucubin-relatively rich chemo-types. These findings provide a solid basis to establish good agriculture practice and select geo-authentic crude drug for *P. major*.

Moreover, the established method was considered suitable for fingerprint analysis to control the quality of *P. major* (RSD of RRT and RPA were not than 15%). This suggested that the method used chromatographic fingerprint combining similarity hierarchical clustering analysis and target peaks quantitative expression may afford consistent discrimination of *P. major* populations based on chemical components profiling as a tool for chemo-taxonomy.

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