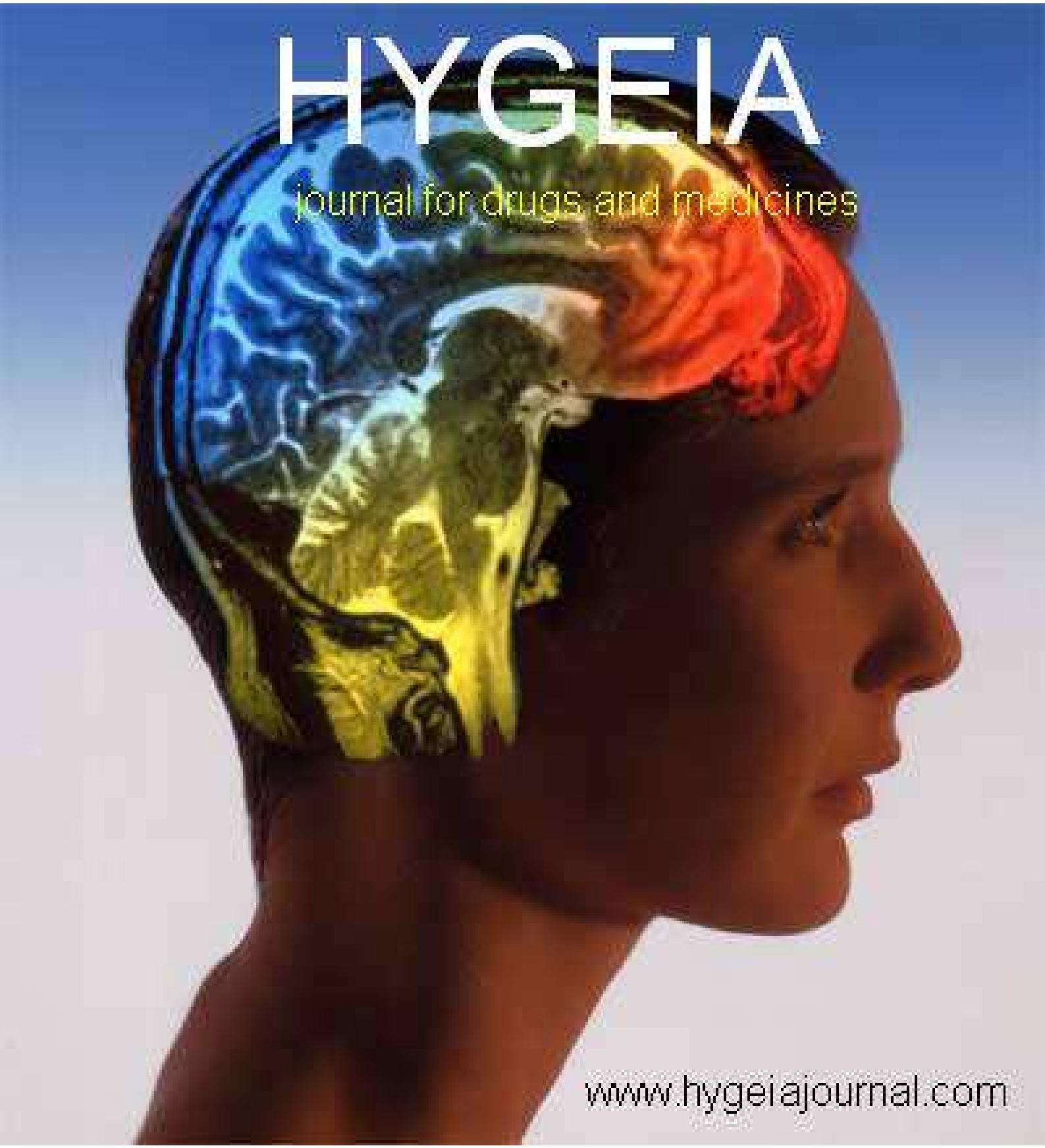
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Stem Cells in Drug Discovery: Current trends and Emerging Challenges

Senthil Kumar Pazhanisamy[1] *, Vinu Jyothi[1, 2]

1 .Pathology and Laboratory Medicine, School of Medicine, Medical University of South Carolina, Charleston, SC 29425, USA.
2 .Pediatric Hematology/Oncology, School of Medicine, Medical University of South Carolina, Charleston, SC 29425, USA.

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Abstract:

Current development in stem cell research ultimately revolutionizes the way drug discovery and development will be directed in the future and also paves the way for innovative cell based therapies in regenerative medicine. The unique and exquisite feature of both embryonic and adult stem cells can be harnessed to continually derive human somatic cell types *in vitro* which otherwise is difficult to generate from other sources. Recently, enormous attention has been directed towards the identification, generation, characterization and application of hESCs-derived tissue precursor cells. Such potential resources and strategies provide unparalleled opportunities in disease modeling, drug discovery, drug development, toxicology, safety assessment, and cell replacement therapies. This review illustrates underlying mechanisms by which stem cells are being exploited by various chemical compounds to generate potential cell models for both biopharmaceutical research and regenerative medicine. Here we also summarize various strategies and differentiation techniques for dissemination of stem cell population *ex vivo*.

Key words: embryonic stem cells, adult stem cells, differentiation, drug discovery and development, biopharmaceutical applications.

1. Introduction

Drug discovery in the biopharmaceutical industry is catalyzed by increasing numbers of identified potential drug targets since the advent of human genome sequencing project. Despite a large number of discovered novel drug targets, clinically proven drugs available for the human disorders are abysmally low. In particular, the current trend is such that even for those low numbers of successful drugs do not have unique targets, mostly having common proteins, genes, or pathways as targets. For instance only 43 novel proteins were targeted by more than 100 popular drugs or new molecular entities (NMEs) in 2001. Indeed, a surprisingly small number of, typically not more than 3, novel host targets or therapeutic proteins could be commercialized each year by the entire pharmaceutical industry[1]. For drug discovery and development against human disorders, various animal cell models are exploited at various stages such as target identification, target validation, lead optimization, drug candidate selection, library screening for early hits, leads, pharmacokinetic, and toxicological analysis.

For Correspondence: scendhil@gmail.com
Contact: (843) 792-4874, Fax: (843) 792-0368

One of the major obstacles hindering the drug developmental process is lack of screening systems based on normal human functional models. It is not surprising that many clinically approved compounds after trials fail at patient therapy due to either their inefficiency or unanticipated toxicity or side-effects arising during clinical practice. Conventionally, biopharmaceutical research relies on animal cells or immortalized human cell lines representing human system to test drug efficacy or toxicity. From target identification to library screening, lead optimization and drug selection, various functional assays are conducted for drug discovery and development.

In order to establish valid functional cell models, cell cultures derived from tumor cell lines are transformed to simulate human proteins which in turn could be exploited as drug targets. In particular, an accurate report on the metabolic fate of a given drug is a prerequisite during pharmaceutical drug development process. Although, these functional cell models are robust and reproducible, they still fail to represent or mimic the human cellular system, posing an enormous challenge to precise and efficient drug discovery for human disorders[2]. For instance, the complex functionalities of hepatocytes are not reflected by any currently available *in vitro* model which is metabolically competent. Tissue culture system offers an alternative technique to isolate the cells of interest. However, the major limitation is that they dedifferentiate quickly and possess only limited cell divisions *in vitro*. Hence, these problems are largely attributed to imperfect disease models which do not faithfully represent the human diseases. It is therefore not surprising that the clinical outcome of pharmaceutical compound remains low as animal models may not truly representing or lack adequate similarities to the human cell system. To circumvent these problems, embryonic stem cells offer far reaching implications, allowing us to generate a variety of fully differentiated cells, rendering an efficient and diverse tissue population for various pharmaceutical research purposes on the road to drug discovery[3,4]. This hES (human embryonic stem) cell derived differentiated or dedifferentiated system not only rejuvenates cell therapy in regenerative medicine but also paves the way for meaningful insights of underlying signaling or regulatory pathways that regulate major cellular mechanisms. In this review, we highlight current challenges and future opportunities for stem cell research in drug development and discovery process.

1.1. Therapeutic impact of embryonic and adult stem cells

It is apparent there are no simplistic chemotherapeutic approaches available for most debilitating disorders, such as degenerative disorders, cancers, and relevant tissue damage disorders. This roadblock in the treatment drives an enormous attention in the potential application of stem cells. A defining characteristic of stem cells is their impressive self-renewal potential with long-term differentiation capabilities[5]. Embryonic stem cells (ESCs) derived from early embryo possess nearly unlimited self-renewal capacity and developmental potential to differentiate into virtually any cell types in an organism.

Due to their impressive self-renewal and differentiation potentials, embryonic stem cells (ESCs) and adult stem cells hold great promise in cell and gene therapy applications in the treatment of many disorders[6,7]. Embryonic stem cell technology provide a potential platform to develop novel functional models by expanding pluripotent stem cells and also converting the ESC populations to generate large number of differentiated precursor cells of various tissues[4]. Researchers demonstrate that adult or tissue stem cells can survive, migrate, differentiate, integrate and reconstitute within the transplanted organ system. Particularly, stem cells from various developmental organs, including embryonic, neural, hematopoietic, and induced pluripotent cell system were successfully transplanted for variety of clinical purposes.

Interestingly, the differentiation of ESCs could be specifically and systematically controlled in a reproducible manner to perform various drug screens using normal differentiated human cells for appropriate signal transduction systems.

Recent advances in the identification, isolation, characterization and *in vitro* culture techniques highlight the unprecedented potential of stem cells to cure disorders. Translating these potentials into clinical benefits encounters enormous challenges, including efficient engraftment of stem cells into desired tissue system, maintaining the genetic stability for long course of time, and preventing the oncogenic potential during stem cell proliferation. Through their regenerative capability, adult SCs are able to differentiate into residing tissue to partially restore the function. Stem cell therapy principally involves introducing a new cell into the damaged or diseased tissue to replenish or rejuvenate the organ or tissue system. The ability of stem cells to self-renew, proliferate and differentiate to form a functionally competent tissue offers a great potential to replace the diseased or damaged tissues[6]. Mesencymal stem cells from fetal bone marrow, for instance, are capable of differentiating into not only osteogenic, adipogenic and endothelial lineages, but also hepatocyte-like cells, chondrocytes, muscles, neural, and erythroid cells[8]. Interestingly, their regenerative and tissue repair potential are not restricted to their local milieu but also to tissues of distal organs via pro-inflammatory cytokines and growth factors. Here, the added benefit is that both autologous and allogenic stem cells have no immunoreactivity problems in systemic administration and local transplantation, rendering stem cells as an ideal choice to deliver the genes of interest in gene therapy applications in various tissues. Development of cell specific gene therapeutic approaches are now underway to cure various diseases including premature aging diseases, diabetes, atherosclerosis, hematopoietic, cardiovascular, musculoskeletal, gastrointestinal, pulmonary, urogenital, ocular, neurodegenerative and skin disorders[9]. Stem cells offer great promise in the treatment of variety of diseases ranging from heamatological disorders, cancer, neuro, cardiac and nephrological disorders. Current research is directed at exploiting the adult and embryonic stem cell to treat many disorders including cancer, Type 1 diabetes mellitus, Parkinson's disease, Huntington's disease, cardiac failure, muscle damage and neurological disorders. Stem cell treatment remains the only treatment modality for the cure of chronic lymphocytic leukaemia (CLL)[10]. Stem cell transplantation (SCT) remains the only treatment capable of cure, but has traditionally been associated with very high morbidity and mortality. For more than three decades, leukemic and lymphoma patients were successfully treated by using bone marrow and umblical stem cells. Stem cell therapy has an advantage over conventional radiotherapy and chemotherapy which could largely compromise normal hematopoietic cells while killing cancer cells.

1.2. Differentiation screens

Recently, there has been enormous attention to develop methodologies to direct ES cells to derive more specific and differentiated cell population for developmental biology and degenerative medicine purposes. In particular, screen the library to identify a novel compound to sequentially disseminate ESCs in a controlled manner to yield desirable differentiated cells in tissue-culture environment. In a classical experiment, Jessells et al., demonstrates that a precise gradient of extracellular components could dictate the transcriptions factors to tailor the specific neural cell representing defined stage of neural development (reviewed in[11]). Interestingly, ESCs can be differentiated stepwise into each differentiation level such as a neural progenitor fate then early DA neural progenitor, followed by late DA neuron progenitor, and finally DA neuron.

Similarly, ES cells could either be directed to become pancreatic β cells with initial endodermal induction, then early pancreas, pancreatic endocrine, and then mature β cells secreting insulin (Reviewed in[12]). Another intriguing study demonstrates that human cord blood (UCB)-derived multipotent stem cells regenerated the spinal cord at the injured site accompanied by improved sensory perception and movement upon 5 weeks after cell transplantation[13]. The major advantage of developing gradual differentiating method is they could represent precise target cell population, thus allowing us to develop specific drugs pertaining to the target cells by excluding untoward toxicities or maximum therapeutic benefits.

1.3. hES derived cardiac myocytes

The dissemination of cardiomyocyte precursor from ESCs or adult SCs is invaluable for the development of heart disease models and also can be utilized for repairing damaged heart tissue *in situ*. hES cells could be directed to establish a large number of cardiomyocytes for cardiac drug discovery, development of novel therapeutics for heart diseases, cardiac safety assessment and cardiac modeling.By screening a large number of chemicals, Takahashi and colleagues demonstrated that the putative use of a small molecule, ascorbic acid to enrich the cells with cardiac phenotype which display spontaneous, rhythmic contractile activity, along with presence of cardiac genes such as sarcomeric myosin and alpha-actinin, GATA4, alpha-MHC, and beta-MHC[14]. Similar screening process in a large combinatorial library by Wu and colleagues identified cardiogenol A-D as a potential differentiating agent to derive more specific cardiomyocytes[15]. Some differentiating compounds such as 5-aza-deoxycytidine are unique to hES to differentiate into cardiomyocytes and fail at mouse ES cells[16]. Studies also demonstrate that both iPS and ES cell-derived cardiomyocytes display cardiac functionality and the beta-adrenergic and muscarinic signaling cascade responses exploit them as an autologous cell source for cellular cardiomyoplasty, and myocardial tissue engineering[17].

1.4. Hepatocytes derived from ESCs

At present, liver transplantation is the only effective treatment for severe liver disorder. However, the liver transplantation therapy is severely limited by shortage of donor organs, operative damage, and the risk of immune rejection. This potential problem profoundly catalyzes the demand for alternative approaches such as cell therapies which offer restoration of liver mass and function. Therefore, hESCs are scalable and have the potential to provide an unlimited supply of replacement somatic cells, which possess significant advantages over their adult stem cell counterparts[18]. For last few years, embryonic stem (ES) cells are being widely studied as a promising source of hepatocytes with their proliferative, renewable, and pluripotent capacities.

Direct differentiation approaches use a two-dimensional tissue culture approach employing extracellular matrixes, growth factors, cytokines, and hormones to facilitate the formation of three-dimensional structures termed embryoid bodies (EBs) which consequently differentiate to varying levels of hepatocyte-like cells (HLCs)[19]. In recent years, hESC differentiation to HLCs has been modified with efficient and functional hepatocyte differentiation demonstrated by several groups[20,21]. Recent study by Hay et al. demonstrates that Wnt3a is differentially expressed at critical stages of human liver development to promote the clonal efficiency of hESCs exhibiting functional hepatic differentiation *in vivo*.

Although there has been major progress in the field, there is still the requirement to select HLCs from other contaminating cell types and undifferentiated stem cells in final cell preparations. Recent reports offer significantly improved yields of HLCs to be used in the modeling of human liver development, disease, transplantation, and drug toxicology for cell based therapies. There are other potential methods available to enrich the functional hepatic progenitor cells. For instance, by using the fluorescent activated cell sorting (FACS) for the asialoglycoprotein receptor method, human HLCs through EBs which secreted functional human liver-specific proteins observed in primary human hepatocytes with human hepatocyte cytochrome P450 metabolic activity[22]. Study using purified adult rat primary adult liver stem cells trans-differentiated into pancreatic endocrine hormone-producing cells when cultured in a high-glucose environment[23]. These results indicate that these hepatic stem cells can differentiate in a non-lineage-restricted manner to trans-differentiate into endocrine pancreas which could be directed for future therapies of diabetes. Moreover, these ESCs and derived hepatocytes could be exploited for a variety of potential pharmaceutical applications. For instance, safety and toxicology assessment, using ESC derived hepatocytes for drug metabolism, usage of differentiated cells to identify of surrogate biomarkers, utilizing genotyping the ESCs for varying responses to drugs due to genetic variations and to explore the underlying mechanisms predisposition to disorders, employing transgenic animal models to process target validation and drug discovery.

1.5. Neurons derived from ESCs

For the development of neuronal drug discovery models, it is crucial to enrich derived neural subtype cells from ESCs and optimize the specific culture conditions. For the treatment of Parkinson's disease, human neural precursor cells could be successfully enriched to generate mid-brain dopaminergic phenotype from GABAergic phenotype[24,25]. In this, robustly generated midbrain dopamine neurons from the hES cells were exploited in preclinical models of Parkinson's disease. This experimental system also offers a renewable source of functional human DA neurons for drug screening and development of cell-replacement strategies for disorders affecting the DA system and to explore the molecular mechanisms that control the development and function of human midbrain DA neurons. One potential strategy by which ESCs could be instructed to commit themselves to a particular lineage cell population is to express nurr-related protein 1 (Nurr1) to enrich dopaminergic neurons (sonic hedgehog homologue (SHH), FGF2 and FGF8)[26]. Similar chemical screening of a large number of compounds identified retinoic acid and an activator of sonic Hedgehog signaling as differentiating agents for embryoid bodies generated from ES cells to ultimately generate functional l motor neurons[27]. Relevant differentiation screening approaches were aimed at producing more differentiated specific cell lines by using chemical factors yielded desired cells at target cells for specific disorders[28, 29].

1.6. Small molecular compounds in stem cells

Several research groups have carried out chemical screening for small compounds to modulate self-renewal in ES, neural stem and other adult stem cells. Underlying mechanisms by which certain small molecules regulate the self-renewal in stem cells have been explored to characterize the molecular signatures of self-renewal and to develop potential therapeutics. Retinoic acid, for instance, leads to alterations in HOX gene expression during embryogenesis and is a modifier of the WNT-mediated signaling pathway[30,31].

Prostaglandin E2 (PGE2), a small lipid mediator, has also recently been shown to regulate HSC self-renewal during embryogenesis and can enhance HSC engraftment, as measured by the competitive repopulation studies in mice[32]. Self-renewal can be augmented in stem cells with self-renewal potential. HSCs can execute the self-renewal programme, but the addition of WNT3A, sonic hedgehog (SHH), and angiopoietin-like factors or PGE2 can increase the size of stem-cell pool.

Some low molecular compounds such as ascorbic acid, retinoic acid, 5-azacytidine and glucocorticoids can remodel the adult tissues preferentially through regulating adult stem cell differentiation. For instance, 5-azacytidine is known to induce mouse mesenchymal progenitors to differentiate[33]. Some cancer cells in dedifferentiated state could be differentiated into less potent cells. These drugs potentially target stem cells to differentiate by suppressing their signaling molecules, and cell cycle mediators. This mechanism paves the way for many pharmaceutical agents such as imatinib (marketed as *Gleevec*; Novartis[34]) bortezomib (*Velcade*; Millennium pharmaceuticals[35]) and geldanamycin[36]. Although, the clinical therapeutic applications remain to be validated, these small molecular compounds offer a great platform to mechanistic understanding of underlying stem cell pathways. Growing number of investigations were successfully employed to screen small compounds that largely influences differentiation characteristics of ES cells.

For instance, a large combinatorial chemical library was subjected to phenotypic cell-based screen to identify diaminopyrimidine compounds (cardiogenol A-D) which selectively and efficiently induce mouse embryonic stem cells (ESCs) to differentiate into cardiomyocytes[37]. It is now increasingly evident that screening small molecules to induce ESC differentiation provides more opportunities to derive a variety of progenitor population[38]. By screening a focused active 2,4-disubstituted-pyrrolopyrimidines library, Ding et al. identified GSK-3 which could differentiate neurons in both mouse embryonic carcinoma and ES cells[39].

1.7. Dedifferentiation screens

Both differentiation and dedifferentiation of adult and embryonic stem cells are rapidly transforming concepts, only partly understood at present. Hence, their defining characteristics and the differences between them perhaps should require further study. By screening a chemical library of diverse compounds culminated in the identification of a microtubule-disrupting molecule, reversine was identified that directs the myeblasts to generate mesenchymal stem cells that could ultimately differentiate into a large numbers of bone and adipose cells. Recent study demonstrates that cell-based screen of chemical libraries provides identification of small molecules that control the self-renewal of ES cells. By using this screening method, SC1 an uncharacterized heterocycle, was discovered which propagates murine ES cells in an undifferentiated, pluripotent state under chemically defined conditions in the absence of feeder cells, serum, and leukemia inhibitory factor.

This methodology potentially expands the number of long-term murine ES cells to derive primary germ layers *in vitro* and in vivo by down-regulating the RasGAP and ERK1[40,41]. These small compounds not only deliver therapeutic advantages of stem cells, but also shed light on novel insights into the underlying molecular mechanisms of stem cells.

1.8. Induced pluripotent stem cell (iPS) cells

Induced pluripotent stem (iPS) cells display pluripotent stem cell characteristics which are artificially derived from adult non-pluripotent cells, by reprogramming their gene expression patterns ex vivo. The generation of human Induced pluoripotent cells (iPS) from human somatic cells revolutionizes the way how regenerative medicine progresses in recent years. Intriguingly, differentiated cells can be reprogrammed to an embryonic-like state by using a defined set of transcription factors to reverse their lineage passage back to a pluripotent state associated with ESC-like phenotype. In a ground breaking study, Shinya Yamanaka's group retrovirally introduced four transcription factors: Oct 3/4, Sox2, c-Myc, and Klf4 in both mouse and human fibroblasts to reprogramme the somatic cells[42]. This technology paved way for unparallel opportunities in regenerative medicine as iPS cells could differentiate into specific progenitor cell types. As it circumvents the use of embryonic stem cells, iPS technology offers a great alternative for the source of differentiated human cells for cell therapeutics in regenerative medicine.

In past decades, gene therapeutic trials against various genetic disorders have not been clinically successful, owing to the paucity and poor quality of adult stem cells in the bone marrow of patients. Now, a combination of gene therapy and induced pluripotent stem (iPS) cell technology could deliver promising therapeutic approaches for the various disorders including fanconi Anemia (FA), cystic fibrosis, and other relevant human genetic diseases. In an elegant experiment, defective genes in cells from patients were rectified using gene therapy[43].

Those repaired cells were then reprogrammed into induced pluoripotent stem cell (iPS) cells using a combination of transcription factors, OCT4, SOX2, KLF4 and cMYC. The resulting FA-iPS cells were indistinguishable from human embryonic stem cells and iPS cells generated from healthy donors, which successfully ameliorate FA phenotype. Importantly, stem cells aspirated from the bone marrow of three CF patients were transfected with Maloney murine leukemia virus carrying CFTR gene[44]. The resulting *ex vivo* co-culture system allows marrow stromal stem cells (MSCs) to differentiate into airway epithelial cells and restores long term functionalities.

This suggests that *ex vivo* gene therapy offers potential advantages such as screen, reprogram, and manipulate the cells before actually delivering them to patients. Similarly, induced pluripotent stem (iPS) cells can be generated from patients with type-1 Diabetes by reprogramming their adult fibroblasts with three transcription factors (OCT4, SOX2, KLF4)[45]. These derived cells, termed DiPS cells have the pluripotent characteristic and therefore can be differentiated into insulin-producing cells.

Interestingly, introduction of microRNAs (miRNAs) – the unique posttranscriptional modulators specific to embryonic stem cells profoundly enhances the production of mouse induced pluripotent stem (iPS) cells. The miRNAs miR-291-3p, miR-294 and miR-295 promote the reprogramming efficiency by Oct4, Sox2 and Klf4 to dedifferentiate the somatic cells into iPS cells[46]. Further analysis of the targets of the miRNAs identified here may offer insights into the reprogramming mechanism. Studies delineate that these ESCs specific miRNAs are highly expressed in ES cells, where they accelerate the cell cycle transition. Various investigations demonstrate that miRNAs regulate the pluripotency of ESCs such that genetic deletion of key miRNA processing enzymes Dicer[47] lose their pluripotency and show defective differentiation perhaps via indirect down-regulation of Oct4, Rex1, Sox2, and Nanog genes.

Besides, murine ESCs with Dicer-deficient mutant ESCs can be partially rescued by the miR-290 cluster miRNAs that downregulate Oct4 indirectly[48]. Current studies are directed towards delineating underlying mechanisms by which reprogramming machineries dictate the somatic cell into pluripotent cell. A growing number of studies are now focusing on iPS cells derived from various patients to offer novel interventions for different diseases, including Type I diabetes, Parkinson's, and Muscular Dystrophy.

1.9. Stem cell mediated prodrug drug delivery

One of the potential problems associated with stem cell transplantation is adverse inflammatory responses in host animal to contradict the therapeutic benefits. To circumvent this problem, recent study designs microencapsulated stem cells in which genetically engineered neural stem cells (NSCs) are delivered in a time-controlled manner. More interestingly, the encapsulated system could also efficiently be programmed to regulate the rate and extent of proliferation and migration of the NSCs. Adult stem cells could be exploited as potential targeted drug delivery system for anticancer drug as they have the tendency to migrate to distal, diseased, and metastatic cancerous tissues. In principle, well-designed NSCs display both the ability to differentiate *in vivo* in a controlled fashion and to sustain their self-renewal, propagation and expansion capabilities at the target sites. NSCs should be immortalized to avoid the transformation into cancer stem cells. In this, stem cells tender great advantage over other therapies as traditional cancer therapeutics are facing increasing difficulties to access the remote and inaccessible cancerous sites in various tissues. For instance, human fetal primary stem cells generate the tumor-targeting neural cell line, HB1.F3.C1, which were then programmed to secrete a form of rabbit carboxylesterase (rCE)[49], which in turn activates an anti-cancer prodrug *Campto/Camptosar* (irinotecan; Pfizer). This study also demonstrates that administration of modified NSCs followed by *Camptosar* profoundly enhances survival rate to almost 100% in mice bearing cancer.

In particular, gene-directed enzyme prodrug therapy (GDEPT) is based on the delivery of a gene that encodes an enzyme which is non-toxic per se, but is able to convert a prodrug into a potent cytotoxin. MSCs can be employed as a vehicle for Prodrug gene therapy to deliver the candidate genes encoding enzymes that convert nontoxic prodrugs into toxic anti-metabolites. Human adipose tissue-derived mesenchymal stem cells (AT-MSC) with enhanced tumor tracking properties provide an attractive opportunity for targeted transgene delivery into the sites of tumor formation and also serve as a potential source of autologous stem cells[50,51]. In this system, Cytosine Deaminase (CD), HSV-1 Thymidine kinase and carboxyesterase genes render sensitivity to anticancer drugs 5-fluorocytosine 5-FC, ganciclovir (GCV) and camptothecin-11 (CPT-11), respectively. The potentiating effect of prodrug 5-FC observed in a recent investigation by Kucerova et al. suggests that human adipose tissue-derived mesenchymal stem cells (AT-MSCs) could be used as a cellular vehicle for CD:UPRT gene (CDy-AT-MSC) to suppress the HT-29 tumor cells *in vitro*[52]. Interestingly, engineered CD-AT-MSCs combined with 5-FC were efficiently controlled human colon cancer xenograft growth *in vivo.*

Besides, this CDy-AT-MSC/5FC augmented the bystander effect and selective cytotoxicity on A375 human melanoma, glioblastoma, HT29 colon, MDA-MB-361 breast cancer cells and bladder carcinoma targets *in vitro*. Similarly, AT-MSC (TK-MSC) expressing Herpes simplex virus - thymidine kinase (HSV-tk) could exert cytotoxic effect on tumor cells upon treatment with prodrug ganciclovir (GCV)[53]. AT-MSC (TK-MSC) displayed both bystander cytotoxic effect on tumor cells and prodrug ganciclovir conversion-mediated suicide effect on TK-MSC.

This supports the idea that mesenchymal stem cells could be utilized for tumor-targeted cancer gene therapy. Due to extensive tropism of neural stem cells (NSC) toward malignant gliomas, NSCs could target medulloblastoma and be used as a cellular therapeutic delivery system which disseminates therapeutic agents to medulloblastoma.

The HB1.F3 cells (an immortalized, clonal human NSC line) were engineered to secrete the prodrug activating enzyme Cytosine Deaminase (CD) and allowed to target medulloblastoma. In this, CD enzyme converts non toxic substrate antifungal agent 5-FC to antitumor agent 5-fluorouracil (5-FU), allowing newly generated 5-FU diffuse into target the surrounding medulloblastoma cells and melanoma brain metastases models[54]. The results confirm the potential clinical utility of these cells and the CD gene as a cell-directed approach for enzyme-mediated prodrug conversion in the field of molecular cancer chemotherapy.

2. Other relevant Pharmaceutical applications

The use of embryonic stem cells for cell-replacement therapy in diseases like diabetes mellitus requires methods to control the development of multipotent cells. Cell therapeutic strategies for long have been exploiting variety of stem cell technologies to gain major benefits. Though, there are several strategies employed to generate pancreatic islet cells, only the strategy using forced expression of PAX4 was successful in promoting the development of insulin-producing cells *in vitro*[55]. Here, the constitutive expression of Pax4 influences ES cells to differentiate into pancreatic lineage, which leads to the formation of islet-like spheroid structures that produce increased levels of insulin. By inhibiting the intracellular signaling regulator PI3-K, pancreatic β-like cells were developed from mouse embryonic stem cells. Although not identical to pancreatic islets of Langerhans, these cells produced significantly higher level of insulin, and displayed glucose-dependent insulin release *in vitro*. They enhanced the circulating insulin levels, controlled weight loss, improved glycemic control, and dramatically rescued survival in mice with diabetes mellitus. These observations demonstrate that embryonic stem cells can serve as a repository of insulin-generating tissue for cell replacement therapy in diabetes mellitus. In co-culture with endothelial cells, embryonic neural progenitor cells (NPCs) show reduced neurogenesis and elevated self-renewal. The adult neural stem cells could even produce progeny that exhibited an endothelial phenotype with enhanced barrier properties. The co-culture of endothelial cells, pericytes and astrocytes adopt the anatomical condition of the blood-brain barrier (BBB) *in vivo*. This set-up can be used as a model of the BBB to study the pharmacokinetics of several neurological drugs which typically transport across the barrier[56,57]. Similar studies were directed to generate ESCs-derived membrane model with ABC efflux pumps to assess the membrane permeability of certain pharmacological agents.

3. Toxicity studies

Due to the limited availability of precise human cell or tissue models *in vitro*, drug toxicity investigations are preferentially carried out in other animal models which typically lead to inaccurate results or misinterpreted toxicology outcomes. To test carcinogenicity of various genotoxic as well as nongenotoxic carcinogens, the Syrian hamster embryo (SHE) cell transformation assay is the only available option which often yields imprecise toxicology outcomes. Although hindered by ethical roadblocks in the past decades, many investigations are now preferentially using either human or mouse embryo cells for embryo cell test (EST)[58]. Since the advent of high-throughput screens during the drug discovery phase, a large number of lead candidates are being selected for drug development.

This catalyzes an enormous need for *in vitro* alternative test models to determine the pharmacokinetics and toxicology profile of compounds in the late- and/or early-development phase. In particular, embryotoxic or teratogenic new chemical entities (NCEs) could be implemented for reproductive toxicology studies. An ECVAM (European Centre for the Validation of Alternative Methods) validated system[59], embryonic stem cell test (EST) utilizes the differentiating potential of murine embryonic stem (ES) cells to test embryotoxicity *in vitro*[60].

4. Conclusions and future directions

Despite the fact there has been tremendous progress in our understanding of stem cells in the past few years, stem cell therapeutics is still a young field such that there are many intriguing aspects of stem cells are still remain to be elucidated to fully understand the therapeutic potential of stem cells. Importantly, mechanisms by which low molecular compounds, signaling pathways, and *ex vivo* culture conditions regulate stem cell behavior is still poorly understood. In this context, unraveling the molecular mechanisms of stem cells is a prerequisite to optimize their therapeutic potentials in drug discovery and development. Recent insights into the differentiation of embryonic, adult, and induced pluripotent stem cells offer great benefits but also raise several fundamental questions in regard to their clinical applications. It is evident that major challenges still remain in deriving potential hESCs by exploiting the iPS technology; how somatic cells fate is reversed into lineage non-specific iPS stem cells and how stem cells reciprocate to certain signals are yet to be answered. It is also fascinating to know how an organism drives stem cell mobilization and their reestablishment at distal tissue organs in response to variety of stress signals. Given that complex degenerative disorders persist despite the conventional therapies further propagates our immense interest in the development of novel strategies based on stem cell therapeutics. Taken together, it is increasingly apparent that combinatorial, multifaceted, and sophisticated approaches should be directed to gain more insights of the stem cells to develop most-promising targeted therapies for various chronic degenerative disorders.

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Formulation and evaluation of polyherbal gels for antinociceptive Activity

D. Varghese [1*], N. Sabarwal P [2], Mane. N.[3] Jonwal .S. Jain[4]

1, 2, 3, 4: Smriti College of Pharmaceutical Education, Indore-452010, Madhya Pradesh, India-452010

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Abstract

In the present study three medicinal plants *Vitex negundo* Linn (Verbenaceae), *Bryophyllum pinnatum* Linn (Crassulaceae) and *Centella asiatica* (Umbelliferae) reported to have significant analgesic potential were selected to be formulated as polyherbal gels with and without permeation enhancers and were investigated for their antinociceptive potential. The dried finely ground herbs were extracted in distilled water and combined in different ratios and incorporated in 5% gel base of carbopol 934 (GF1-GF15). Antinociceptive activity was evaluated using two animal models, viz. acetic acid induced writhing response and formalin induced hind paw licking and was compared against control and standard diclofenac diethylamine gel (1.16%). All the formulations significantly ($P<0.001$) reduced the number of abdominal constrictions and stretching of hind limbs induced by the injection of acetic acid. The topical application of polyherbal gel formulations GF1-GF15 significantly ($P<0.001$) inhibited the licking time induced by the injection of formalin. The study established these polyherbal gels as suitable alternatives to synthetic analgesic agents.

Keywords: Antinociceptive, gel, polyherbal, writhing

1. Introduction

Herbal medicine has become an integral part of standard healthcare based on combination of traditional usage and ongoing scientific research. Burgeoning interest in medicinal herbs has increased scientific scrutiny of their therapeutic potential and safety[1]. Natural products and medicinal plants are believed to be an important source of new chemical substances with potential therapeutic efficacy[2]. *Vitex negundo* Linn (Verbenaceae), a large aromatic shrub with bluish purple flowers has been used for various medicinal purposes in Ayurvedic and Unani systems of medicine. Analgesic and anti-inflammatory effects of *Vitex negundo* have been reviewed thoroughly[3].

* For correspondence: Email: dhanilav@yahoo.co.in
Contact: +91 9424513942; Fax: +91 731 2802467

Presence of various flavonoid compounds like 5-hydroxy-3,6,7,3',4'-pentamethoxy flavone and 3,5-dihydroxy-3',4',6,7-tetramethoxy flavonol are reported in the leaves of *Vitex negundo*[4]. *Bryophyllum pinnatum* Linn (Crassulaceae) is a shrub with simple or trifoliate petiolate leaves used mainly in folk medicine to alleviate pains of various intensities and etiologies. The analgesic potency of the aqueous extract of the leaves of *Bryophyllum pinnatum* demonstrated strong analgesic potency comparable in a time and dose-dependent manner to a nonsteroidal anti-inflammatory drug. Presence of various flavonoids, polyphenols, triterpenoids are reported in the leaves of *Bryophyllum pinnatum*[5]. *Centella asiatica* (Umbelliferae) is a perennial, herbaceous creeper used in traditional medicine in the treatment of inflammation, anemia, asthma, blood disorders, bronchitis, fever, urinary discharge and splenomegaly. Various bioactive terpene acids such as asiatic acid and madecassic acid are reported from the water-methanol extraction of *Centella asiatica*. These phytocompounds may be present in the crude extract of *Centella asiatica* that may account for the antinociceptive and antiinflammatory activities[6]. The objective of the present investigation is to formulate topical gels containing these herbs with and without permeation enhancers and screen their antinociceptive potential.

2. Materials and methods

2. 1. **Plant Material**

The leaves of *Bryophyllum pinnatum* were collected locally from Indore in the month of July 2008. The dried leaves of *Vitex negundo* and dried leaves of *Centella asiatica* were purchased from the local market and the above herbs were identified by comparing with standard herbarium specimens available in AICRP on Medicinal & Aromatic plants, J.N. Krishi Vishwa Vidyalaya, College of Agriculture, Indore, Madhya Pradesh. Voucher specimen (SCOPE/Phcog/08/11(a-c)) of the plant was deposited in the Herbarium of the Department of Pharmacognosy, Smriti College of Pharmaceutical Education Indore, Madhya Pradesh.

2. 2**. Preparation of aqueous extract**

The powdered leaves of *Bryophyllum pinnatum*, *Vitex negundo* and *Centella asiatica* were soxheleted individually with distilled water and the percentage yields obtained were 15.6%, 14% and 21% respectively.

To find the most effective combination for a polyherbal gel formulation, the obtained extracts were mixed in different proportions as defined in Table 1.

2. 3. **Preparation of polyherbal gel formulations**

Polyherbal gel formulations were prepared by incorporating the extract combined in different ratios in 5% gel base of carbopol 934. Menthol and oleic acids in concentration of 2.5% and 5% were incorporated in the gels as permeation enhancers. Glycerin 5% w/w was incorporated as humectant, methyl paraben 0.02 % w/w as preservative, triethanolamine q.s. for pH in the range 6-7 and distilled water q.s. up to 100 ml. The formulation code of different extract combinations with and without permeation enhancers are given in Table 2.

2. 4**. Animals**

Swiss albino mice of either sex weighing 20-25 gm, from Veterinary College, Mhow (Madhya Pradesh) were used for these studies. The animals were maintained at 28±2^{o}C at a relative humidity of 50-55% and at 12 h light and dark cycles. Animals were housed in groups of six per cage and were fed with standard pellet diet *ad libitum* and allowed free access to drinking water. The animals were acclimatized to laboratory conditions prior to experimentation. All experimental protocols were approved by Institutional Animal Care and Ethics Committee headed by CPCSEA (Committee for Purpose of Control and Supervision of Experiments on Animals).

2. 5**. Antinociceptive Activity**

Two models, viz. acetic acid induced writhing response and formalin induced hind paw licking using albino mice were employed to study the antinociceptive effect of the polyherbal gel formulations. The animals were divided into seventeen groups of six animals each. Group I served as control and received 1% v/v acetic acid intraperitoneally, group II served as standard group and was topically treated with diclofenac diethylamine gel (1.16%), groups III- VII were treated with polyherbal gels (GF1-GF5) with extract combination (1:2:3), groups VIII-XII (GF6-GF10) and groups XIII- XVII (GF11- GF15) were treated with polyherbal gels with extract combination (2:3:1) and (3:1:2) respectively. In all the treatment groups the polyherbal gel with different combination of extracts, with and without permeation enhancers were topically applied on the abdomen region 15 minutes prior to administration of acetic acid 1%v/v. In formalin test standard and polyherbal gel formulations were topically applied on the dorsal surface of the hind paw of mice 30 min prior to administration of dilute formalin.

2. 6**. Acetic acid induced writhing response**[7]

Acetic acid (1% v/v) was administered intraperitoneally to all the groups at the dose of 1 ml/kg body weight 15 min after the application of polyherbal gels. A writhe is indicated by abdominal constriction and full extension of hind limb. The number of abdominal constrictions (writhing) and stretching with a jerk of the hind limb was counted for 30 minutes after administering acetic acid. Percent protection against writhing movement was taken as index of antinociception. Antinociceptive activity was expressed as the percentage inhibition of abdominal constrictions between control animals and mice pre-treated (n=6) with the polyherbal gel formulations using the formula
(Control mean – Treated mean) x 100 / Control mean

2.7. **Formalin induced hind paw licking in mice**[8]

Nociception was induced by injecting 20µl dilute formalin (1% in saline solution) under the skin of the dorsal surface of the hind paw of the mice. Each mice was challenged with formalin 30 minutes after being pretreated with the standard and test polyherbal formulations GF1-GF15 and then placed into a transparent plastic cage and observed. The licking response was monitored until 30 minutes starting immediately after the injection of formalin. The amount of time spent licking the injected paw was considered as indicative of pain. Antinociception was defined as a statistically significant reduction in the time spent in licking the injected paw in comparison with the control group during 30 min.

3. Statistical analysis

The statistical analysis of all the results was carried out using one-way ANOVA followed by Tukey Kramer multiple comparison tests. All the results obtained in the study were compared with the control group. P values <0.001 were considered to be statistically significant.

4. Results and discussion

Preliminary screening of aqueous extracts of leaves of *Bryophyllum pinnatum*, *Vitex negundo* and *Centella asiatica* showed significant analgesic potential in accordance with the findings of other workers. The topical application of polyherbal gel formulations GF1-GF15 significantly inhibited the writhing reaction induced by acetic acid. All the formulations significantly (P<0.001) reduced the number of abdominal constrictions and stretching of hind limbs induced by the injection of acetic acid as compared to control (Table 3). GF5 and GF10 exhibited the highest writhing inhibition percentage of 95.43% and 93.38% respectively with oleic acid 5% as the permeation enhancer. The highest writhing inhibition percentage was found in the formulations where *Centella asiatica* and *Vitex negundo* were taken in higher concentrations.

The topical application of polyherbal gel formulations GF1-GF15 significantly inhibited the licking time. All the formulations significantly (P<0.001) reduced the paw licking induced by the injection of formalin as compared to control. The percentage protection in formalin test is shown in Table 4. GF3 exhibited the highest percentage protection of 77.60% with menthol 5% as the permeation enhancer.
Flavonoids are known to inhibit the enzyme prostaglandin synthetase. Since prostaglandins are involved in the pain perception and are inhibited by flavonoids, it could be suggested that reduced availability of prostaglandins caused by flavonoids in *Vitex negundo*[4] and *Bryophyllum pinnatum*[5] might be responsible for the analgesic effect. Similarly the terpene acids found in *Centella asiatica* might also be responsible for the antinociceptive effects[6].

The primary role of the skin is to act as a barrier for drug absorption. There is ample proof that the stratum corneum is the primary barrier to drug absorption through skin[9]. Menthol is a natural skin permeation enhancer with relatively low skin irritancy. Mechanism of skin permeation enhancement by menthol can be attributed to the increase in skin flux by altering the barrier properties of the stratum corneum[10]. Oleic acid a mono-unsaturated fatty acid, because of its kinked or twisted shape and structure, is supposed to insert itself into the lipid matrices of the skin, widening the non-aqueous channels, and thereby increasing permeation[11]. Menthol and oleic acid both enhanced the skin permeation of the polyherbal gels.

5. Conclusion

The polyherbal gels with highest concentration of *Vitex negundo* and *Centella asiatica* demonstrated significant antinociceptive potential. Menthol and Oleic acid in a concentration of 5% also enhanced the antinociceptive effect when compared with gels with no permeation enhancers.

Table 1: Combination of extracts for polyherbal gel formulations

S.No.	Code	Ratio		
		Bryophyllum pinnatum	***Vitex negundo***	***Centella asiatica***
1.	F1	1	2	3
2.	F2	2	3	1
3.	F3	3	1	2

Table 2: Formulation of different extract combinations with and without permeation enhancers

Code	**Extract combination (*Bryphyllum pinnatum:Vitex negundo: Centella asiatica*)**	**Permeation enhancers**	
		Menthol	**Oleic acid**
GF1	1:2:3	-	-
GF2	1:2:3	2.5	-
GF3	1:2:3	5.0	-
GF4	1:2:3	-	2.5
GF5	1:2:3	-	5.0
GF6	2:3:1	-	-
GF7	2:3:1	2.5	-
GF8	2:3:1	5.0	-
GF9	2:3:1	-	2.5
GF10	2:3:1	-	5.0
GF11	3:1:2	-	-
GF12	3:1:2	2.5	-
GF13	3:1:2	5.0	-
GF14	3:1:2	-	2.5
GF15	3:1:2	-	5.0

Table 3: Effect of polyherbal gels on acetic acid induced writhing in mice.

Groups	Number of contractions	Percentage inhibition
Control	73.0 ± 0.96	0.00
Standard	28.5 ± 1.40*	60.95
GF1	34.66 ± 1.33*	52.52
GF2	12.5 ± 0.95*	82.87
GF3	6.16 ± 0.98*	91.56
GF4	17.83 ± 0.54*	75.57
GF5	3.33 ± 0.49*	95.43
GF6	41.0 ± 0.36*	43.83
GF7	18.66 ± 0.49*	74.43
GF8	13.0 ± 0.73*	82.19
GF9	22.66 ± 0.55*	68.95
GF10	4.83 ± 0.6*	93.38
GF11	39.16 ± 0.79*	46.35
GF12	15.16 ± 0.47*	79.23
GF13	12.0 ± 0.57*	83.56
GF14	24.16 ± 0.60*	66.90
GF15	8.5 ± 0.76*	88.35

n=6, the values are expressed in Mean ± SEM; * = $p < 0.001$ when compared with control group (One way ANOVA followed by Tukey's multiple comparison tests).

Table.4 Antinociceptive effect of polyherbal gels on formalin test in mice.

Groups	Mean licking time	Percentage protection
Control	61.0 ± 0.36	0.00
Standard	20.83 ± 0.4*	65.85
GF1	38.33 ± 0.21*	37.16
GF2	20.16 ± 0.30*	66.95
GF3	13.66 ± 0.33*	77.60
GF4	17.0 ± 0.25*	72.13
GF5	14.66 ± 0.33*	75.96
GF6	42.16 ± 0.47*	30.88
GF7	21.83 ± 0.30*	64.21
GF8	14.33 ± 0.21*	76.50
GF9	19.33 ± 0.42*	68.31
GF10	15.33 ± 0.42*	74.86
GF11	45.83 ± 0.30*	24.86
GF12	25.66 ± 0.33*	57.93
GF13	17.5 ± 0.56*	71.31
GF14	24.16 ± 0.47*	60.39
GF15	23.16 ± 0.47*	62.03

n=6, the values are expressed in Mean ± SEM; * = $p < 0.001$ when compared with control group (One way ANOVA followed by Tukey's multiple comparison tests).

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Pharmacognostical Studies on the Bark of *Artocarpus hirsutus* Lam.

Dibinlal D[1*], Sathish Sekar D[2], Senthil Kumar KL[3]

1, 2, 3: Department of Pharmacognosy, Padmavathi College of Pharmacy, Periyanahalli, Dharmapuri, Tamilnadu, India

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1. Abstract

*Artocarpus hirsutus Lam.,(*Wild jack) belonging to the family Moraceae a large evergreen tree up to 70m height, found up to an altitude of 1200M in evergreen India. The wood is straight blackish brown in color; it is very strong tree and has main advantage of lightness. It is used for the treatment of ulcers, diarrhea and pimples. The present study includes Pharmacognostical studies of the bark of Artocarpus hirsutus Lam.

Key words: *Artocarpus hirsutus, Pharmacognostical, Sectioning,*

2. Introduction

The plant *Artocarpus hirsutus* Lam, (Moraceae) [1, 4] is a large evergreen tree upto 70m in height, found up to an altitude of 1200M, in evergreen forest of peninsular India. The outer colour of bark is grey and inner colour is brown. The leaves are elliptic rhomboid or ovate and dark green in colour. Male head narrowly cylindrical, female heads simple. Seeds are long ovoid. It distributed in southern part of India. It requires heavy rainfall probably notes than 175cm.The wood is light straight or interlocked-grained and even tentured. Its colour is blackish brown. The heartwood is almost as strong; contain all type of flavanoid except nor-artocarpin. The main property and uses of unripe fruits are sour, astringent, sweet, thermogenic, indigestible, an aphrodisiac, constipating and cause flatulence. An infusion of the bark is applied to cure small pimples and cracks on the skin. Powered bark is used to heal sores. Dry leaves are useful in treating bubose and hydrocele.

*For Correspondence: dibinlal@gmail.com
Contact: +91 9846535114

For over viewing medicinal properties of the plant, my effort was to study Pharmacognostical parameter of the bark of *Artocarpus hirsutus* Lam.

3. Materials and methods[5-7]

3.1. Collection of specimens

The plant specimens for the proposed study where collected from Kollam district, Kerala, care was taken to select healthy plans and normal organs. The required samples of different organs were cut and removed from the plant and fixed in FAA (Formalin 5ml+Acetic acid 5ml+70% Ethyl alcohol 90ml). After 24 hours of fixing the specimens were dehydrated with grader series of tertiary butyl alcohol as per the schedule given by Sass, 1940. Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point58-60^{0}C) until TBA solution attained supersaturation.The specimens were cast into paraffin blocks. The voucher specimen no. is PARC 2009/411.

3.2. Sectioning [8]

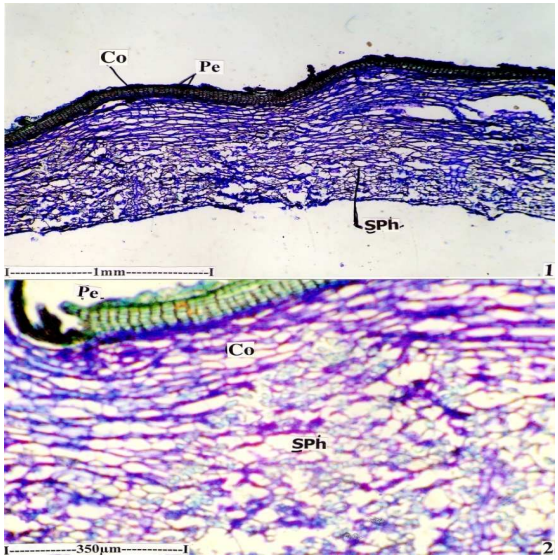
The paraffin embedded specimens were sectioned with help of rotary microtome. The thickness of the section was 10-12 µm. dewaxing of the section was customary procedure (Johnsen 1940).the section were stained with toluedene blue as per the method published by O´brien et al(1964).Since toludine blue is a polychromatic stain. The staining results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. where ever necessary sections were also stained with safranin and fast green and IKI(for starch).

3.3. Photo micrographs[9]

Microscopic descriptions of tissues are supplemented with micrographs where ever necessary. Photographs of different magnifications were taken with Nikon lab photo 2 Microscopic unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of figures are indicated by the scale-bars. Descriptive terms of the anatomical feature are as given in the standard anatomy books. (Esau 1964).

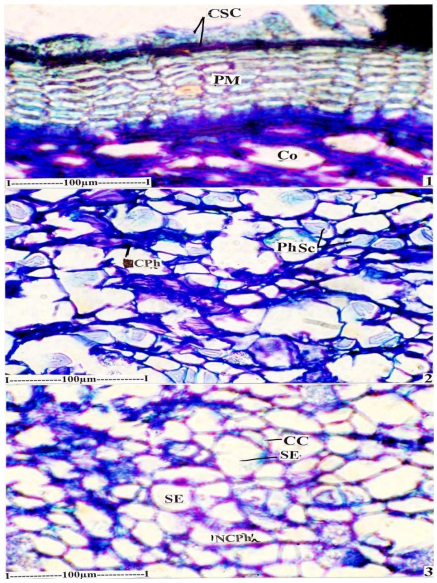
4. Microscopical characters

Figure1.1 T.S of bark-entire view
Figure1.2 T.S of bark-Periderm wit Secondary Phloem enlarged



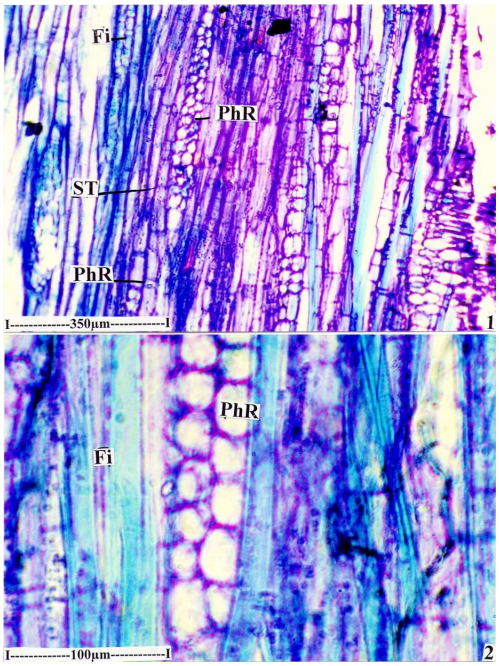
(**Co**-Cortex, **Pe**- Periderm, **SPh**-Secondary Phloem)

Figure 2.1 T.S of bark – Outer Phellem and Cortex
Figure 2.2 T.S of bark- Collapsed Phloem
Figure 2.3 T.S of bark- Non Collapsed Phloem



(**CC**-Companion Cells, **Co**-Cortex, **CSC**-Crushed Surface Cell, **CPh**- Collapsed Phloem, **NCPh**- Non Collapsed Phloem, **PhSc**-Phloem Scleroids, **PM**- Phellem, **SE**-Sieve Elements.)

Fig 3 TLS of phloem
Fig 3.1 TLS of Phloem showing FIbres phloemrays Sieve Tube members
Fig 3.2 TLS of Phloem showing Fibers Phloem rays Sieve Tube members enlarged



(**Fi**- Fibre, **PhR**-Phloem Rays, **ST**-Spongy Mesophyll Tissue).

Powder microscopy of the bark

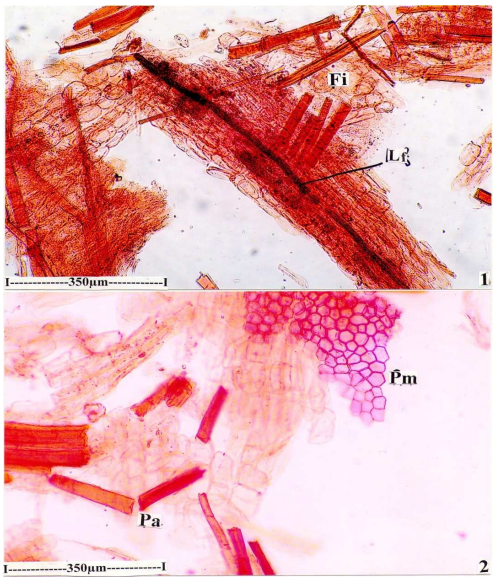
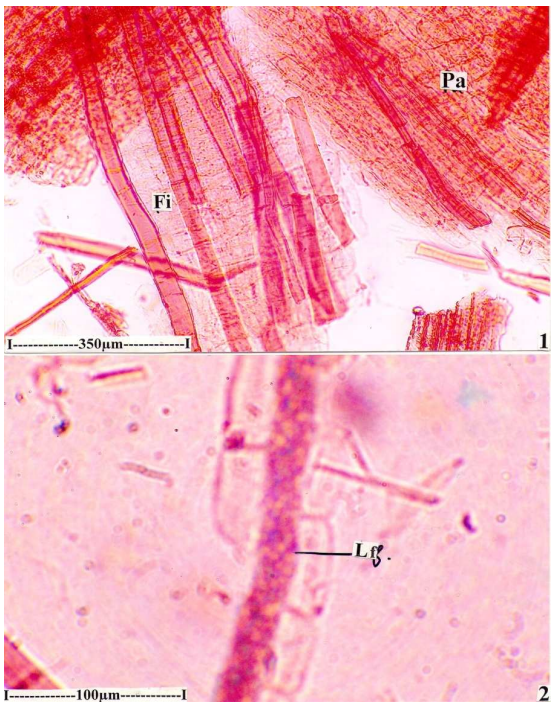


Fig 4.1 Laticefer and Fibers in the bark powder
Fig 4.2 Parenchyma cells and fragment of Phellem cells

(**Fi**-Fibers, **Lf**-Laticefer, **Pa**-Parenchyma, **Pm**-Phellem)

Figure 5.1.Fibres with Parenchyma cells
Figure5.2 Laticefer magnified



(**Fi**-Fibers, **Pa**-Parenchyma, **Lf**-Laticefer)

Results and Discussion[10-15]

The bark of the lateral branch is thick and scaly. It is 850-900µm thick, somewhat wavy superficial periderm and inner zone of secondary phloem (fig1.1).On the surface of the periderm is seen a dark layer of crush and obliterated epidermal and sub epidermal cells. The dark surface layer tends to break and liberated small fragments on the surface (fig 2.1). The periderm has only phellem cells and phelloderm layers are not evident. The phellem has 9 or 10 layer of tangentially oblong, tubular cells with suberised wall. There the tangential walls are straight, but the radial walls are varies.

Secondary phloem is wider than the periderm. In between the periderm and secondary phloem is a narrow zone of cortex where the cells compressed and tangentially elongate. (Fig 1.1 and 1.2)

The secondary phloem can be differentiated into outer or collapsed phloem, and inner is non collapsed phloem. (fig 2.2, 2.3). The collapsed phloem consists of crushed dark thick irregular lines of phloem elements and isolated scattered sclerenchyma elements. (fig2.2) The non collapsed phloem has intact phloem elements, isolated scleranchyma are located in the non collapsed phloem (fig2.3)

TLS View Of Bark (fig 3.1, 3.2)

In tangential longitudinal section, the bark shows phloem rays, phloem sclerenchyma and sieve element. The rays are mostly uniseriate or biseriate (having single vertcular rows or two vertical rows cells). The rays are heterocellular having wider cells at the end and squqrish swollen cells in the middle.(fig3.1). Average length of rays is 550 µm and breadth is 40 µm.

Phloem sclerenchyma includes long, wide, thick walled and less lignified fibres. (fig 3.2). They are straight solitary lines. Phloem parenchyma cells are vertically oblong and occur in vertical shades (fig3.1)

Powder Microscopy

The bark powder shows the following inclusions.

Laticefers (fig 4.1, 5.2)

These are long narrow and wide unbranched canal like tubes which are the laticefers. These cells are latex secreting tubes, scatter in vertical orientation in the bark. The content of the laticefers is glandular and dence (fig5.2). The laticefers are non-separate and anastomosing.

Phellem cells (fig 4.2)

Small piece of polygonal compact parenchyma cells are seen the powder. The pieces are the phellem cells of the periderm seen in surface tubes.

Phloem fibres (fig 5.1)

Fibers of broken cuts are abundant in the powder. They have thick walls and wide lumen, measuring 30-40µm diameter. No pits are seen on their walls.Verticulary elongated rectangular parenchyma cells are also common in the powder; these cells have thick walls and wide lumen (fig4.2). The parenchyma cells have no special inclusion.

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Amelioration of CNS Toxicities of L-Dopa in Experimental Models of Parkinson's disease by Concurrent Treatment with *Tinospora cordifolia*

Shanish Antony A[1*], Partha DebRoy[2], Vadivelan R[3], Jaysankar K[4], Vikram M[5], Nandini S[6], Sundeep M[7], Elango K[8], Suresh B[9]

1,2,3,4,5,6,7,8,9 :Department of Pharmacology and Toxicology, J. S. S. College of Pharmacy, Ootacamund, Tamilnadu.

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Abstract

Parkinson's disease is the second most common neurodegenerative disease, primarily affecting people of ages over 45-55 years, although young adults and even children can also be affected. The gold standard drug for the treatment of Parkinson's disease is L-DOPA, but various studies have proved that the treatment with L-DOPA leads to the death of surviving dopaminergic neurons in the CNS[1]. Hence we have approached to counteract the toxicities of L-DOPA therapy by co-administration of *Tinospora cordifolia* crude powder. On the zero day each animals were given with an intraperitoneal (ip) injection of 1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) (20mg/kg) and after 48 hours the animals were treated with L-DOPA or L-DOPA plus *Tinospora cordifolia* crude powder upto 30 days. At the end of the study period we were evaluated the level of anxiety, grip strength and mitochondrial complex-I activity. The results revealed that the co-administration of *Tinospora cordifolia* crude powder protected the dopaminergic neurons when compared with Sham operated control group. In conclusion, we would like to state that the treatment with *Tinospora cordifolia* crude powder could reduce the toxicities of L-DOPA therapy for Parkinson's disease.

Keywords: Parkinson's disease, CNS toxicities, L-DOPA, *Tinospora cordifolia*, MPTP

1. Introduction

Parkinson's disease (PD) is the most common neurologically based movement disorder, clinically diagnosed by the presence of bradykinesia, postural instability, resting tremor and rigidity[2]. PD occurs when a group of cells in an area of the brain called the substantia nigra (SN) begin to malfunction and die[2].

*For Correspondence: shanishantony@yahoo.com
Contact: +91 934321803

These cells in the SN produce a chemical called dopamine. Dopamine is a neurotransmitter, or chemical messenger, that sends information to the parts of the brain that control movement and coordination. When a person has Parkinson's disease, their dopamine-producing cells begin to die and the amount of dopamine produced in the brain decreases[2]. As we enter the new century, Parkinson's disease ranks among the most common late life neurodegenerative diseases, affecting approximately 1.5% to 2.0% of the population older than age 60[3].

The causes are still largely unknown. Current thinking is that major gene mutations cause only a small proportion of all cases and that in most cases; non-genetic factors play a part, probably in interaction with susceptibility genes. Numerous epidemiological studies have been done to identify such non-genetic risk factors, but most were small and methodologically limited[4, 5].

Medications can help manage problems with walking, movement and tremor by increasing the brain's supply of dopamine. The most effective Parkinson's drug is L-DOPA, it passes into the brain and is converted to dopamine. L-DOPA is combined with carbidopa to create the combination drug Sinemet in Europe and L-DOPA is combined with a similar substance, benserazide[6].

In prolonged L-DOPA therapy, the apparent buffering capacity is lost and the patient’s motor state may fluctuate dramatically with each dose of the drug, a common problem is the development of wearing off phenomenon: each dose of L-DOPA affectively improves mobility for a period of time, about 1 or 2 hours, but rigidity and akinesia return rapidly at the end of dosing interval[6]. Increasing the dose and frequency of administration can improve this situation, but this often is limited by the development of dyskinesias, excessive and abnormal involuntary movements[7]. Dyskinesias are observed most often when the plasma L-DOPA concentration is high although in some individuals dyskinesia or dystonia may be triggered when the level is rising or falling. These movements can be as uncomfortable and disabling as the rigidity and akinesia of PD.

In the later stages of PD, patients may fluctuate rapidly between being "off", having no beneficial effects from their medications and being "on" but with disabling dyskinesias, a situation called on/off phenomenon[6]. In addition to motor fluctuations, several other adverse effects are observed after prolonged L-DOPA treatment. A common one is the induction of hallucinations and confusion, particularly common in the elderly. Conventional anti psychotic agents such as phenothiazines are effective in L-DOPA induced psychosis but may cause worsening of Parkinsonism through their actions at the dopamine D_2 receptor [3, 8, 9]. Facial tics, grimacing and mild anxiety, nightmares etc. to severe depression, mania these are also some common side effects of L-DOPA therapy[6].

L-DOPA can generate free radicals during its own oxidation as well as during oxidative metabolism of its product, dopamine. Thus, it appears rational to propose that an excessive quantity of free radicals is generated, and this may be one of the factors which contribute to the side-effects of L-DOPA therapy[1]. Therefore, it is possible that supplementation with appropriate multiple antioxidants may improve the efficacy of L-DOPA therapy[1]. With these supporting evidences it is clear that the L-DOPA toxicity can be attenuated by co-administration of one good antioxidant or a drug which can facilitate the actions and activities of mitochondrial complex-I which is an integral component in Parkinson’s disease [1]. *Tinospora cordifolia* (TC), family: Menispermaceae, has been extensively studied and reported to have potent antioxidant activity[10], literatures report that, if taken regularly in high doses; it has no major side effect and toxicity[10] and it seems TC may bear a potential use in neurodegenerative disease affecting the cerebral neurons[11].

The active adaptogenic constituents present in TC are diterpene compounds including tinosporone, tinosporic acid, cordifolisides A to E, syringen, the yellow alkaloid, berberine, giloin, crude giloininand, a glucosidal bitter principle as well as polysaccharides, including arabinogalactan polysaccharide[12]. Among these berberine ameliorated renal dysfunction in streptozotocin-induced diabetic rats, which was accompanied by inhibition of renal aldose reductase and reduction of oxidative stress[13]. Anti-tumor promoting action of berberine is attributed to its antioxidant property[14]. In the view of above points, we selected *Tinospora cordifolia* crude powder (TCCP) which is wide reported for potent antioxidant[10] and free radical scavenging activities for reducing the toxicities of L-DOPA therapy in experimental PD.

2. Materials and methods

Plant material

Tinospora cordifolia (TC), family: Menispermaceae, were collected from local areas of Coimbatore district, Tamilnadu. The collected areal parts of TC were authenticated from Botanical survey of India, Agriculture University, Coimbatore, Tamilnadu, India. For further reference a voucher specimen has been deposited at J. S. S. College of Pharmacy herbarium, ootacamund, India. *Herbarium accession no*: JSSCP/ P Cog/ 137.

Chemicals

The chemicals which were used for the present study were procured from Sd-Fine Chemicals Mumbai, Sigma Aldrich USA, Loba chemicals Mumbai, Merk chemicals Mumbai.

Preparation of plant material[15]

Aerial parts were cut in to small pieces and dried in sun light. Then they were powdered carefully by using mechanical blender. The powdered drug was finely sieved and kept in air tight container. A further unique advantage of *Tinospora cordifolia* is that it is effective when given orally[16]. So we have selected oral route for the administration of TCCP with 0.3% w/v of carboxy methyl cellulose (CMC) solution as solvent through oral gavage tube.

HPTLC Standardization of Tinospora cordifolia using Tinosporaside[17]

Around 20g of air dried sample was ground to pass through 20 mesh SS sieve and 5g from it was accurately weiged and refluxed with 50ml of methanol about 2h. The resulting solution was filtered, concentrated under vaccum, redissolved in methanol and the final volume was made up to 50 ml. This solution was used for HPTLC analysis.

A camag HPTLC system equipped with a sample applicator Linomat IV, twin rough plate development chamber, TLC scanner III and integration software CATS 4.0 was used. An aluminum plate (10X10 cm) precoated with silica gel 60F 254 (E. Merck) was used as an adsorbent, toluene, acetone and water in the ratio of 5: 15: 1 were used as a mobile phase. The solvent was allowed to run up to 80 mm and the chromatograms were scanned at 220 nm. A 0.5 mg/ml solution of Tinosporaside – reference standard was prepared in methanol as a stock solution. The test solution was shaken well and 15 µl was applied on a TLC plate along with 1, 2, 4, 8 and 16 µl of standard Tinosporaside, likewise three such plates were prepared.

The plates were developed up to 80 mm under chamber saturation condition. After air drying the solvent, the plates were scanned at 220 nm in UV reflectance mode. The amount of Tinosporaside present was determined using the calibration curve plotted between concentration and area of standard. The regression equation was found to be, Y = 7.087X + 107.744 with correlation coefficient of 0.9911. The content of Tinosporaside were quantified and percentage recoveries were calculated (Table 1). By this method the Rf of Tinosporaside was about 0.58. The content of Tinosporaside was found to be 0.05% w/w in sample.

3. Acute toxicity study of TCCP (OECD guide line: 423)[18]

Female Wister rats of weight (180-220g) were taken for the study and kept for overnight fasting. Next day, body weight was taken and TCCP was administered orally at a dose of 2000 mg/kg in 0.3% CMC. Then the animals were observed for mortality and morbidity at 0, 1/2, 1, 2, 4, 6, 8, 12, and 24hours.
Feed was given to the animals after 4 hours of dosing and body weight was checked 6 hours after dosing. Morbidity like convulsions, tremors, grip strength and pupil dilatation were observed. The animals were observed twice daily for 14 days and body weight was taken. The same experiment was repeated once again on 3 female rats (preferably female) as there was no observable clinical toxicity for the animals on the phase 1 study. From acute toxicity study, 200 mg/kg (1/10 of tested dose) of TCCP was selected as dose[11].

Animals

Healthy, adult Wister rats of both sexes (180-220g) were obtained from central animal house facility from J. S. S. College of pharmacy, Tamilnadu, India. Animals were cared for in accordance with guiding principles for the care and use of Animals approved by committee for purpose and control for the supervision and experimentation on animals and Institutional Animal Ethics Committee.*CPCSEA approval number:* JSSCP/ IAEC/ M. PHARM/ PH. COLOGY/ 09/ 2008-09.

Animals were divided into four groups of three male and three female rats in each group.
Group I: Control (normal) group
Group II: Sham operated control (MPTP treated) group
Group III: MPTP + L- DOPA (9 mg/kg) treated group
Group IV: MPTP + L – DOPA + TCCP (200mg /kg) treated group

Induction of Parkinsonism by MPTP[19, 20, 21]

On zero day each animal were given an intraperitoneal (ip) injection of MPTP (20 mg/kg) in normal saline. MPTP (1-Methyl-4-phenyl-1,2,3,6-tertahydropyridine) is a neurotoxin, which after absorption converted to MPP^+ radical which specifically degenerate dopamine-producing neurons in the SN a part of a mid brain. Due to degeneration of dopaminergic neurons the amount of dopamine production will reduce and leads to Parkinsonism. Then forty-eight hours after the induction of Parkinsonism the animals were treated orally with L- DOPA or L-DOPA plus TCCP at 09.00 hours up to 30 days, 0.3% w/v of carboxy methyl cellulose (CMC) solution was used as vehicle.

4. Pharmacological Evaluation

4.1. **Anti-anxiety activity**[22]

The elevated plus maze was used to evaluate the anti-anxiety effect in animals. The apparatus consists of four compartments, two open and two enclosed compartments. After placing animals individually in the centre of the maze, head facing towards the open arm, the stop watch was started and noted down the following parameters for 5 min for each animal. First preference of animal to open or enclosed arm (An arm entry defined as the entry of four paws in to arm). Average time each animal spend in each arm was calculated as, total duration time in arm divided by number of entries. Then compared the percentage preference of animal to open /enclose arm, average time spent in open arm and number of entries in open arm for each group.

4.2. **Muscle grip strength study**[23]

The main symptom of the Parkinson's disease is muscle rigidity. The loss of muscle grip is an indication of muscle rigidity. This effect can be easily studied in animals by using rotarod apparatus. Twenty rpm was selected as an appropriate speed .The animal was placed individually one by one on the rotating rod. Noted the 'fall off time' when animal falls from the rotating rod. Then the fall off time of animal in control and all treated group was compared.

5. Molecular pharmacological evaluation

5.1. **Isolation of mitochondria from rat mid brain**[24]

Tissue was homogenized with a Dounce tissue grinder in mitochondrial isolation buffer (70 mM sucrose, 210 mM mannitol, 5 mM Tris HCl, 1 mM EDTA; pH 7.4) and suspensions were centrifuged at 800 g, 4°C, for 10 min. The supernatant fluids were centrifuged at 13000 g, 4°C, for 10 min, and the pellets were washed with mitochondrial isolation buffer and centrifuged at 13000 g, 4°C, for 10 min to obtain the crude mitochondrial fraction.

5.2. **Estimation of Complex-I activity**[24]

NADH: ubiquinone oxidoreducase (Complex-I) activity was measured in the SN as described in the literature. Brain mitochondria, isolated as above, were lysed by freeze–thawing in hypotonic buffer (25 mM KH_2PO_4, 5 mM $MgCl_2$, pH 7.4). The reaction was initiated by the addition of 50 µg mitochondria to the assay buffer (hypotonic buffer containing 65 µM ubiquinone, 130 µM NADH, 2 µg/ml antimycin A and 2.5 mg/ml defatted bovine serum albumin). The oxidation of NADH by Complex-I was monitored spectrophotometrically at 340 nm for 2 min at 30°C. The activity was monitored for a further 2 min following the addition of rotenone (2 µg/ml). The difference between the rate of oxidation before and after the addition of rotenone was used to calculate Complex-I activity.

6. Statistical analysis

The collected datas were subjected to appropriate statistical tests, one-way ANOVA (Analysis of Variance) followed by Bonferroni multiple comparisons test, nonparametric repeated measures ANOVA followed by Dunnet's multiple comparisons test. P values of less than 0.001 were considered highly significant. The analysis was carried using Graph pad Instat software of version 3.

7. Results

7.1. Effect of L-DOPA and L-DOPA plus TCCP on anxiety behavior

When compared with control group, the MPTP treated group showed more preference to open arm. But, when compared with MPTP treated group, the L-DOPA and L-DOPA plus TCCP treated groups showed less preference to open arm and it indicated that, the treatment with TCCP did not alter the activities or functions of Dopaminergic neurons and could have maintained the dopamine concentration in CNS. The more preference to open arm shows less anxiety, dopamine levels and anxiety are directly proportional.

7.2. Effect of L-DOPA and L-DOPA plus TCCP on muscle grip strength

When compared with control group the retention time was significantly reduced for MPTP treated group. But when compared with MPTP treated group, the L-DOPA and L-DOPA plus TCCP treated groups showed more retention time. The results suggested that retention time or muscle co-ordination was improved by either L-DOPA or L-DOPA plus TCCP treated groups. The data indicated that the treatment with TCCP can facilitate the muscle coordination with some actions on dopaminergic neurons.

7.3. Effect of L-DOPA and L-DOPA plus TCCP on Complex-I activity

The Complex-I activity were estimated from mitochondrial fractions isolated from brain tissue homogenate. When compared with control animals the mitochondrial activity was significantly reduced for MPTP and L-DOPA treated group. But the concurrent treatment with TCCP had significantly retained the Complex-I activity.

8. Discussion

There is growing evidence that oxidative stress and mitochondrial respiratory failures with attendant decrease in energy output are implicated in nigral neuronal death in PD[2]. However it is not known, which cellular elements (neurons or glial cells) are major targets of oxygen-mediated damage. L-DOPA therapy is one of the common therapies for advanced PD. But, the severe side-effects of this therapy appear in about five years[7]. The reasons for this are not known; however, L-DOPA can generate free radicals during its own oxidation as well as during oxidative metabolism of it product, dopamine[25, 26, 27]. Thus, it appears rational to propose that an excessive quantity of free radicals is generated, and this may be one of the factors which contribute to the side-effects of L-DOPA therapy. Selegiline used in combination with

L-DOPA may reduce free radical levels by reducing the oxidative metabolism of dopamine; however, it would not affect the level of free radicals generated by the oxidation of L-DOPA. Earlier studies suggested that supplementation with appropriate multiple antioxidants may improve the efficacy or reduce the toxicity of L-DOPA therapy[26, 27].

With these supporting evidences it is clear that the L-DOPA toxicity can be attenuated by co-administration of one good anti-oxidant or a drug which can facilitate or unaffected the activities of mitochondrial complex-I which deteriorate during L-DOPA therapy. With this concern we have evaluated the effect of TCCP for reducing the toxicities of L-DOPA.

We could evaluate the parameters such as level of anxiety, grip strength and mitochondrial Complex-I activity. The level of anxiety was comparably none for L-DOPA or L-DOPA plus TCCP treated groups. The MPTP treated groups, showed less anxiety and it was comparably reduced when compared with normal control. These findings indicate that, the TCCP treatment did not alter the role or action of L-DOPA in experimental animals. The concept of anti-anxiety test was introduced to assess whether TCCP treatment have any interference with L-DOPA affects action to regain dopamine level in dopamine depleted animals.

From the results of muscle grip strength, it is clear that L-DOPA and L-DOPA plus TCCP did not alter muscle co-ordination. This study suggested and further supports that TCCP treatment did not alter the affects of L-DOPA therapy in experimental PD. Earlier reports evidenced that L-DOPA therapy leads to mitochondrial degeneration and leads to neuronal cell death. At last we estimated the mid brain mitochondrial Complex-I activity and the activity was unaffected by TCCP treatment.

The Complex-I activity in L-DOPA treated group was drastically reduced and it is the valuable finding that L-DOPA treatment could lead to further degenerative effect in surviving dopaminergic neurons. Two consistent biochemical 'signatures' in the SN of idiopathic PD cases are a deficiency of mitochondrial Complex-I and abnormally high levels of free-radical damage[2]. These two events are thought to be interrelated, because inhibition of Complex-I activity can increase free radical production, and increased free radical production impairs Complex-I activity. Studies that have attempted to modify the disease course in PD by reducing free-radical formation have shown relatively modest effects on PD[3]. This is valuable information in this research that the protection of mitochondria by TCCP could solve the toxicity of L-DOPA therapy. From the results it may be clear that the co-administration of TCCP can reduce the toxicities of L-DOPA which is a gold standard drug for Parkinson's treatment.

Apart from this, a different plant Mucuna prurita, family: Fabaceae, seeds contain high concentrations of L-DOPA, it has long been used in traditional ayurvedic Indian medicine for the treatment of Parkinson's disease, but the seed may cause birth defects and has uterine stimulant activity during pregnancy, contraindicated in combination with M AO inhibitors, potentiate androgenic medications, potentiate insulin and antidiabetic medication and prolonged use lead to neurotoxicity as conventional L-DOPA treatment[28].

9. Conclusion

In the conclusion, we would like to state that the treatment with TCCP can reduce the toxicity of L-DOPA therapy for Parkinson's disease. The mitochondrial activity retained by TCCP showed a promising way for the usefulness of TCCP for the treatment of clinical Parkinson's disease. The further pharmacological and clinical investigations are needed to implement it for clinical use.

Table 1. Recovery of Tinosporaside from *Tinospora cordifolia*

Sample	**Amount Fortified (mg/ml)**	**Observed Value (mg/ml)**			**Calculated value (%)**	**Average Recovery s(%)**
TC	0.5	0.997	0.089	0.098	5.10	
TC	0.5	0.451	0.484	0.493	4.65	95%
TC	0.5	0.981	0.933	0.942	2.68	

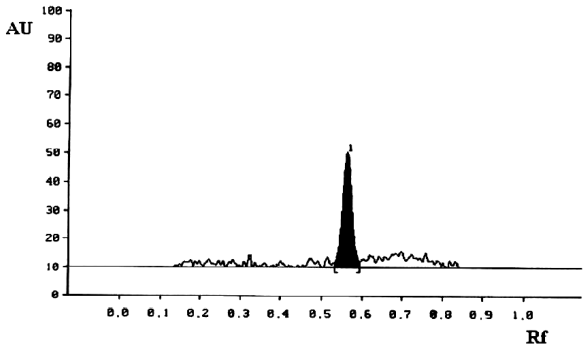

Fig. 1: HPTLC chromatogram of standard Tinosporaside (Rf = 0.58).

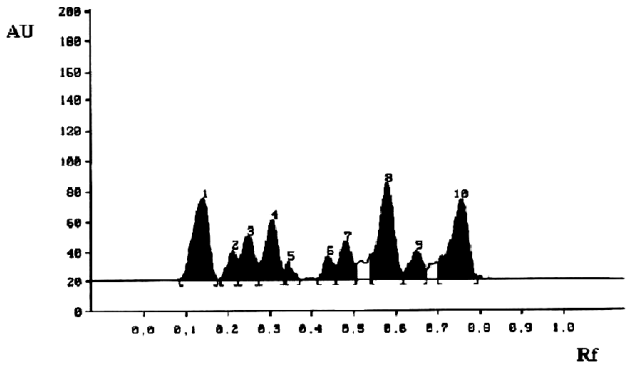

Fig. 2: HPTLC chromatogram of *Tinospora cordifolia* test sample (Rf = 0.58).

Table 2. Effect of L-DOPA and L-DOPA plus TCCP on anxiety behavior

Group	**Average time spent in arm(sec)**		**%Preference to open arm**
	Open arm	**Enclosed arm**	
Control	7.220 ±1.800	58.1122±3.224	10.452±2.0018
Only MPTP treated	14.800 ±1.029	30.7781±2.929	48.220±4.5221***
MPTP + L-dopa	9.812±2.334	59.1195±3.288	12.4187±0.114^{ns}
MPTP + L-dopa + TCCP	8.552±1.770	60.296±3.1102	11.224±1.3890^{ns}

Values are mean ±SEM; n=6 in each group, ***P<0.001 when compared to normal control, ^{ns}P>0.05 when compared to normal control (one-way ANOVA followed by Bonferroni multiple comparisons test)

Table 3. Effect of L-DOPA and L-DOPA plus TCCP on muscle grip strength activity

Group	**Retention time(sec)**
Control	120.442±1.437
Only MPTP treated	10.56±1.678$^{###}$
MPTP + L-dopa	65.974±1.531**
MPTP + L-dopa + TCCP	84.623±1.098***

Values are mean ±SEM; n=6 in each group, ***P<0.001 when compared to MPTP treated group, **P<0.01 when compared to MPTP treated group, $^{###}$P<0.001 when compared to control group, $^{#}$P<0.05 when compared to control group. (one-way ANOVA followed by Bonferroni multiple comparisons test)

Table 4. Effect of L-DOPA and L-DOPA plus TCCP on complex-I activity

Group	**Concentration(nmol/min/mg protein)**
Control	91.6055±1.796
Only MPTP treated	39.5692±2.768$^{###}$
MPTP + L-dopa	24.9425±2.7650**$^{##}$
MPTP + L-dopa + TCCP	89.5217±1.6254***

Values are mean ±SEM; n=6 in each group, ***P<0.001 when compared to MPTP treated group, **P<0.01 when compared to MPTP treated group, $^{###}$P<0.001 when compared to control, $^{##}$P<0.01 when compared to control group. (One-way ANOVA followed by Bonferroni multiple comparisons test)

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Herbs Used for Brain Disorders

Sandhya S[1]*, Vinod KR[2], Sravan Kumar[3]

1, 2, 3, Nalanda College of Pharmacy, Nalgonda, A.P. 50800, India

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Abstract

Human brain disorders vary a wide rage which includes Alzheimer's disease, Parkinson's disease, depression, epilepsy, schizophrenia, anxiety, Huntington's disease etc. Psychotherapeutics does not meet properly for therapeutic possibilities for majority of patients with mental health problems but herbal remedies are ultimate therapeutic hope for such patients. Many synthetic drugs because of many unwanted but unavoidable side effects have poor patient compliance. Therefore herbal treatment is being preferred over conventional treatments. Much attention and so scope is drawn towards herbal remedy of many brain disorders. This session covers a broad spectrum of natural drugs used in specific brain disorders. This topic also discusses the source of active constituent and specific part of the plant being used.

Keywords: Reverse pharmacology, Bioavailability, Alzheimer's disease, Parkinson's disease, Depression, Epilepsy, Schizophrenia, and Anxiety.

1. Introduction

Nature is the best combinatorial chemist and possibly has answers to all diseases of mankind. Failure of some synthetic drugs and its side effects have prompted many researches to go back to ancient healing methods which use herbal medicines to give relief. Many of the thousands of plant species growing through out the world have a direct pharmacological action on the body[1-7].Herbal treatment is a natural form of healing or alternative therapy where herbs and plants are used in the form of extracts, pills, syrup or powder to cure ailments or diseases of human beings and in some cases animals too. Today herbal remedies are back into prominence. The efficacy of many conventional medicines which once had near universal effectiveness against serious infections is on the wane.

In Ayurveda-drug discovery uses "Reverse pharmacology", in which drug candidates are first identified, based on large scale use in the population and validated in clinical trials. Till now, natural product compounds discovered from medicinal plants (and their analogues also) have provided numerous clinically useful drugs. Four billion people or about 80% of the world's population uses herbal medicine as part of health care.

*For correspondence: sanpharm@gmail.com
Contact: +91 9010055004

In India itself, there are more than 1100 medicinal plants grown all over the wild forests. Of these, some 60 genuses are used immensely in medicinal preparations[8, 9, 10]. The hydro soluble fiber *plantago ovata* husk improves levodopa with carbidopa bioavailability after administration in Parkinson's disease.

Curcuma oil modulates the nitric oxide system response to cerebral ischemia/reperfusion injury. Nimbolide a limonoid from *azadirachta indica* inhibits proliferation and induces apoptosis of human choriocharcinoma cells in Parkinson's disease.

Effects of a standardized *Bacopamonniera* extract on cognitive performance, anxiety & depression in the elderly. Curcumin has anti convulsant activity on increasing current electric shock seizures in mice[11, 12]. Common brain disorders include Alzheimer's disease, Parkinson's disease, depression, epilepsy, schizophrenia, anxiety and Huntington's disease[13-16].

Alzheimer's disease:

Alzheimer's disease originally defined as prsenile dementia, means an acquired organic mental disorder with loss of intellectual abilities of sufficient seviarity to interfere with social or occupational functioning. It is associated with brain shrinkage and localized loss of neurons, mainly in the hippocampus and basal fore brain. Two microscopic features are characteristic of the disease namely extra cellular amyloid plaques consisting of amorphous extracelluar deposits of β-amyloid protein and intranuronal neurofibrillary tangles, comprising filaments of phosphorilated form of a microtubule associated protein. This disease is also considered as a short term memory loss.

Parkinson's disease:

It is a progressive disorder of movement associated with continuous shivering that occurs mainly in the elderly. It is commonly associated with dementia. The symptoms include tremor at rest usually starting in the hands, muscle rigidity detectable as an increased resistance in passive limb movement, hypokinesis suppression of voluntary muscles. In this condition the neurotransmitter levels are decreased in brain, such as dopamine,5-HT, acetylcholine ,nor epinephrine. These neurotransmitters are decreased mainly in the substantianigra and carpus striatum of brain.

Depression:

Depression is the most common affective disorder which is accompanied by hallucination and delusions. It is a common affective disorder of mood rather than disturbances of thought or cognition. In this disease condition the neurotransmitters levels in brain is increased such as dopamine, acetylcholine, nor epinephrine etc.

The symptoms of this disease are of two types- emotional symptoms: Feelings of guilt, loss of motivation, ugliness etc and biological symptoms: Retardation of thought, loss of libido, sleep disturbance and loss of appetite.

There are 2 types of depressive syndrome namely

1) Unipolar depression: In this mood swings are always in the same direction.
2) Bipolar depression: In which depression alternates with mania.

Epilepsy:

The characteristic event in epilepsy is seizure. This is associated with high frequency discharge of impulses by group of neurons in the brain. It is divided into 1) Partial epilepsy:

In which the discharge begins locally. In which localized areas of brain are damaged; the symptoms depend on the brain region or regions involved and 2) Generalized epilepsy: In which total brain is damaged, including reticular system.

Schizophrenia:

It is one of the most important forms of psychiatric (mental) illness. In this diseased condition patient don't know what is happening at present and he does not cooperate with the physician for treatment.

The symptoms of this disease are 2 types:

1) Positive symptoms: delusions (often paranoid in nature), Hallucination, thought disorders, abnormal behaviour.
2) Negative symptoms: withdrawal from social contact, flattening of emotional responses. In this condition the level of neurotransmitter such as dopamine, 5HT, acetylcholine, nor epinephrine level is increased in the brain.

Huntington's disease [HD]

Huntington's disease is called as Huntington disease, Huntington's chorea, chorea major, or simply HD and is the most common genetic cause of chorea. This incurable, neurodegenerative disorder was named after the American physician George Huntington who accurately described it in 1872. Prevalence, per country, is up to 7 people in 100,000 (in populations of Western European inheritence), and can be much higher in localized regions. Onset of physical symptoms can begin at any age, although the mean age of onset is 35 to 44 years of age. Less commonly, onset is before the age of twenty, and the condition is classified as juvenile HD (also known as akinetic-rigid HD or Westphal variant HD) - which progresses faster with slightly different symptoms. In 1993 genetic testing was made possible with the discovery of a single causal gene, the first non-sexlinked dominant disease gene to be found,as such counselling for HD had to be developed and became a model for other dominant disorders. The test can be performed before the onset of symptoms, at any age - even pre-birth, which has raised various ethical issues and their debate is heated.

The mechanism of the disease is not fully understood, but a number of factors have been identified. A mutation in the Huntingtin gene, causes the production of the mutant protein huntingtin, which in turn produces cell and macroscopical changes in the brain.There is no cure for HD, although there are treatments to relieve some of its symptoms. The most characteristic initial physical symptoms are jerky, random, and uncontrollable movements called chorea. Rigidity and dystonia become evident as the disorder progresses, and gradually become the dominant physical symptoms.

Anxiety:

Anxiety is a psychological and physiological state characterized by cognitive, somatic, emotional, and behavioral components. These components combine to create an unpleasant feeling that is typically associated with uneasiness, fear, or worry. Anxiety is a generalized mood state that occurs without an identifiable triggering stimulus. As such, it is distinguished from fear, which occurs in the presence of an external threat. Additionally, fear is related to the specific behaviors of escape and avoidance, whereas anxiety is the result of threats that are perceived to be uncontrollable or unavoidable.

Different types of herbs for anxiety

Lavender is a herb with properties that is excellent for treating panic and anxiety. It affects the central nervous system in much the same way as some drugs without the negative side effects. Passion flower can help in high blood pressure and when used as herbs for anxiety it can be put in tea or food. It is also an ingredient in many herbal remedies. Ginseng has long been used for anxiety and is a natural immune booster. The Chinese have known this for a long time. Cannabis sativa is usually smoked but can be eaten and is a great anxiety reliever. Many are aware of this and people worldwide are abusing this remedy. Valerian is used throughout the world as a natural sedative and helps with insomnia and panic attacks. It is also a mild painkiller and is considered very safe for short term use. Kava Kava is a root used for anxiety and is also well known in the treatment of sleep disorders such as insomnia. Lemon balm is good for headaches and also for relieving stress and anxiety. It is a natural sedative and is good for easing tension.Chamomille is put into tea and has been a highly touted herb for anxiety[17-21].

2. Details of Plants having CNS activity

1. *Hypericum perforatum*(St.John's wort):

It consist dried aerial parts and flowers of *Hypericum perforatum* Family Hypericaceae (Clusiaceae).Chemical constituents include anthraquinones mainly hypercin and pseudohypercin. The current use of St.John's wort for the treatment of mild to moderate depression. The antidepressant activity of "hypercin" is attributed to inhibition of neuronal uptake of seratonin,nor epinephrine and dopamine like many other antidepressants and also inhibits GABA & glutamate uptake in brain[22,23].

2. *Piper methysticum*(kava-kava):

It consists of dried root of *Piper methysticum* Family Piperaceae. It contains piperdine, kava pyrones. It is used as sedative, anxiolytic and hallucinogen. The active constituents kava pyrones have a variety of actions like inhibition of voltage dependent sodium channels, increasing neither GABA-A receptor density blocking nor epinephrine reuptake and suppressing the release of glutamate[18].

3. Valeriana walchii:

It consists of the dried rhizomes, stolons and roots of *Valeriana walchii*. Family Valerianaceae. It contains sesquiterpenes like balerenal; also contain esters like bornyl formate, euginyl isovalerate, and alcohols, eugenol. It comprises of acids, esters, ketones like faurinone. The mechanism of action of valerian tends to sedate by stimulating activity of the nerve transmitter GABA that dampens the brain arousal system[9].

4. *Ginkgo*(Maiden hair-tree):

Leaves are obtained from the dioeceous tree *Ginkgobiloba* family Ginkgoaceae. It is only living plant in this family containing flavonoid glycoside.

It contains diterpine lactones like ginkgolides A, B, C, J, and M. It is used in symptoms of short term memory in Alzheimer's disease and also in anxiety[9].

5. *Centella asiatica*(Jal brahmi,mandukparani):

It consists of fresh or dried leaves and stems of *Centella asiatica* family Umbelliferae. It contains saponins Asiaticosides also contain Asiatic acid, madacassic acid, betulic acid. Used as anxiolytic agent, used in epilepsy and also considered as antidepressant. It decreases the levels of malondialdehyde (MDA) with simultaneous increase in the levels of glutathione in the brain[22].

6. Withania somnifera (Ashwagandha):

It consists dried roots and stem bases of *Withania somnifera* family Solanaceae. The main constituent is steroidal lactone, withaferin and withanolides. These withaferin A, B, C, also contain active principles of sitoindosides VII, VIII. It has sedative and hypnotic properties. The active principles sitoindosides have shown anti stress activity. Used as antidepressant. Both Ashwagandha and lorazepam group demonstrated reduced brain levels of a marker of clinical anxiety. It also exhibits anti depressant effect comparable to that induced by imipramine in the forced swim induced behavioral despair and "learned helplessness" tests[24].

3. Discussion

Herbal remedy for human brain disorders is much preferred over synthetic drugs because of various side effects of synthetic drugs ranging from sleep disorders to withdrawal syndromes. Herbal treatment not only improves patient compliance but also there are possibilities of enhancing the bioavailability of many drugs. Active constituents extracted from specific parts of various plant origins have proved to be beneficial. Although some formulations have drawn attention, in depth clinical trials should be conducted which will be a major tool to prove the benefits for a patient. That is what matters!

Table 1. Herbal drugs used in Schizophrenia [18, 19]

Sl.no	Plant name	Family	Common name	Mode of use	Chemical constituents
1	Catharanthus rosea	Apocynaceae	Red periwinkle	Dried Root	Indole and indoline alkaloids- ajmalicine, lochnerine, dimeric; indole base of monoterpene- vinblastine, vincristine.
2	Rauwolfia serpentine	Apocynaceae	Sarpaganda	Dried Root	Indole alkaloids- reserpine, ajmaline, serpentine
3	Canscora diffusa	Gentianaceae	Janjada	Fresh whole plant	Beta amyrin, xanthones
4	Datura metel	Solanaceae	Nalla ummetta	Dried whole plant	Tropane alkaloids- hyoscyamine, scopolamine, flavonoids

Table 2. Herbal drugs used in Alzheimer's disease [9, 19, 21, 25]

Sl.no	Plant name	Family	Common name	Mode of use	Chemical constituents
1	Melissa officinalis	Labiatae	Lemon balm	Fresh leaf	Lutiolin(flavonal glycoside)
2	Salvia officinalis	Labiatae	Sage leaf	Fresh leaf	A,β-thujone together with cineole
3	Centella asiatica	Apiaceae	Indian penni wort	fresh root	Aciaticosides(saponins)
4	Catharanthus roseus	Apocynaceae	Periwinkle	Dried root	Vincristine, vinblastine
5	Ginkgo biloba	Ginkgonaceae	Maiden hair tree	Dried leaf	Ginkgolides (A,B,C,J,M)

Table 3. Herbal drugs used in Parkinson's disease [18, 25]

Sl.no	Plant name	Family	Common name	Mode of use	Chemical constituents
1	Blepharis maderaspatensis	Acanthaceae	Nethirs poondu	Dry seed	Steroids- gomisin D
2	Smilax perfoliata	Smilacaceae	Ram damtena	Dry root	Steroidal sapogenins- diosgenin
3	Smilax zeylanica	Smilacaceae	Rough blind weed, hill lotus	Dry root	Alpha, beta hydroxy acids
4	Plantago ovata	Plantaginaceae	Flax seed	Husk fibre	Mucilage, cyano genetic glycocydes- Linamarin, lotaustralin
5	Azadirachta indica	Maliaceae	Neem, margosa	Fresh leaf	Meliacin- nimbolide, quercetin, kaempferol
6	Emblica officinalis	Euphorbiaceae	Amla, Indian goose berry	Fruit	Tannins, Phyllembelin, Pectins, Vitamin C

Table 4. Herbal drugs used in Depression (affective disorder) [12, 22, 23]

Sl.no.	Plant name	Family	Common name	Plant part used	Chemical constituents
1	Dendrophthoe falcata	Loranthaceae	Honey suckle-mistletoc	Fresh/ dried whole plant	Tanins, flavons, oleanolic acid, beta sitosterol, stigmasterol
2	Breynia retusa	Euphorbiaceae	Kanumu chettu	Dried bark	Triacontane, peonidin, lanosterol
3	Celtis philippensis	Ulmaceae	White Indian nettle	Fresh/ dried whole plant	Betulin, di- methyl elagic acid, gallic acid, leucocyanide glycoside
4	Asparagus recemosus	Liliaceae	Water roof,wild carrot, satavari	Fresh tuber	Asperagin, shatavarin
5	Hipericum perforatum	Hypericaceae	St.John's wort	Dried arial parts	Hypercin, pseudo hypercin

Table 5. Herbal drugs used as Sedatives & Hypnotics [1, 24, 25]

Sl.no	Plant name	Family	Common name	Mode of use	Chemical constituents
1	Sonchus oleraceus	Compositae	Sow thistle, milk thistle	Dried Stem	Glyphosphate
2	Xanthium indicum	Compositae	Cocklebur	Dried whole plant	Alkaloids, polyplenolic compounds, saponins,
3	Aganosma dichotoma	Apocynaceae	Nallateega	Dried whole plant	Kampferol, phenolic acid
4	Nicotiana tubacum	Solanaceae	Tobacco	Dried leaf	Alkaloids-nicotine, nornicotine, anablastine

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Toxicological profiles of the leaf extracts of *Wrightia arborea* & *Wrightia tinctoria*

LAKSHMI DEVI .S [1]*, MADHU. C. DIVAKAR[2]

1. *Department of Pharmacognosy, Sri Ramakrishna College of Pharmacy,SRIPMS, Coimbatore,TamilNadu,India,641044*
2. *Crescent College of Pharmaceutical Sciences, Kannur, Kerala, India-670358*

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Abstract

The research work was conducted with the leaf solvent extracts of *Wrightia arborea* and *Wrightia tinctoria* to make toxicological profiles by employing Brine Shrimp Assay method (BSA) (*Artemia Salina LEACH*).. The LC_{50} values were determined for both the plant solvent extracts respectively in µg/ml of active compounds and extracts. It was found that the leaf ethanolic and methanolic extracts were toxic for the Brine Shrimp Naupli. The results indicated that *Wrightia tinctoria* leaf ethanol (70%) extract and methanolic extract showed LC_{50} values of 471.604 and 517.038 µg/ml respectively .While the *Wrightia arborea* leaf ethanol (70%) extract and methanolic extracts showed LC_{50} values of 498.213 and 531.082 µg/ml respectively. The remaining solvent extracts showed no toxicity (as found more than 1000 µg/ml) in BSA method.

Key Words: Wrightia tinctoria, Wrightia arborea, Brine shrimp Assay (BSA), Toxicological profiles.

1. Introduction

Traditional medicine should be able to play an even greater role in the modern primary healthcare system of the developing countries. The natural ingredients of traditional medicine are believed to be more acceptable to the human body with out producing any toxicity on compared to modern synthetic drugs. Thus the most important factor needed is to derive the maximum benefit from the traditional system of medicine for providing adequate health care services to rural people[1]. Many new natural compounds are isolated, characterized and published without any biological testing whatsoever. Even compounds extracted ,from plant extracts would be toxic at higher concentrations Brine Shrimp Lethality Bioassay in a bench top bioassay method for evaluating the toxicity anticancer, antimicrobial and pharmacological activities of natural products.[2,3,4]

*For Correspondance : ldevijp@yahoo.co.in
Contact: +91 9047034536

By this method, natural product extracts, fractions as well as pure compounds can be tested for their bioactivities. The BSA method is indicative of cytotoxicity and a wide range of pharmacological activities of the compounds.[5,6] The eggs of brine shrimp, *Artermia salina* LEACH, are readily available at low cost as a food for tropical fish, and they remain viable for years in the dry state. Upon being placed in a brine solution the eggs hatch within 48 hours, providing large numbers of larvae (nauplii). Brine shrimp have been previously utilized in various bioassay systems. Among these applications have been the analyses of pesticide residues [7, 8] mycotoxins[9, 10] stream pollutants, anesthetics dinoflagellate toxins, morphine – like compounds[11], toxicity of oil dispersants, co carcinogenicity of phorbol esters[12] and toxicants in marine environments. Most workers have made use of the hatched nauplii, although inhibition of hatching of the eggs has also been studied [13, 14].

2. Experimental

2. 2. **Plant Material**

The leaves of *Wrightia tinctoria* and *Wrightia arborea* was authenticated at the Botanical Survey of India and voucher specimen was deposited at the herbarium of the same.

2. 3. **Preparation of Extract**

The leaves of *Wrightia tinctoria* and *Wrightia arborea* family Apocyanaceae were washed dried and ground to a fine powder and soxhleted with ethanol (70%) methanol, water petroleum ether dichloromethane, ethyl acetate and chloroform. The percentage yield of all the extracts were calculated as given in the table.

2. 4. **Preparation of Simulated Sea Water**[2]

38 gm of sea salt was weighed by rough balance and dissolved in 1 liter of double- distilled water in a small tank and then filtered off to get a clear solution. This simulated sea water was used for hatching of brine shrimp.

Simulated sea water was taken in a shallow rectangular dish with a plastic divider which had several 2mm holes, which was clamped in a dish to make two unequal compartments. The eggs were sprinkled into the larger compartment which was darkened, while the smaller compartment was illuminated. The shrimps were allowed for two days to hatch and mature as nauplii (larvae).

2. 5 **.Preparation of test sample**

Samples were prepared by dissolving 50 mg of each extract in 5ml of dimethyl sulfoxide (DMSO) and various concentrations like 0,100,200,400,500 and 1000µg/ml solutions were prepared from the stock solution with the help of a micropipette having 100 units equal to 1 ml.

2. 6. **Bioassay**

In each of the vial containing different concentration of test samples, 10 brine shrimp nauplii were transferred with the help of 9 inches disposable pipette and simulated sea water was added to make 5ml. The nauplii can be counted macroscopically in the stem of the pipette against a lighted background. A drop of dry yeast suspension was added as food to each vial.

The vials were maintained under illumination. Survivors were counted, with the aid of 3x magnifying glass after 1,6,12 and 24 hours. The percentage deaths at each dose and control were determined after 12 hours were taken as standard and from this the percentage mortality/lethality was calculated[18]. By using Abbott's formula % death = [(test- control)/Control] x 100 and the LC_{50} were determined from a dose response graph.

3. Results and discussion

Among the seven different solvent leaf extracts of *Wrightia tinctoria* and *Wrightia arborea the ethanolic* (70%) and methanolic leaf extracts showed an LC_{50} of 471.604 µg/ml and 517.038 µg/ml for *Wrightia tinctoria* and 498.213 µg/ml and 531.082 µg/ml for *Wrightia arborea* .All the other solvent extracts taken for there two leaves showed no toxicity in BSA method.

The percentage mortality LC_{50} was also calculated by constructing graphs considering log concentration versus percentage mortality. Though after 24 hours, all the samples showed a significant lethality but these were not accepted for the safest and more accurate result. Because, after 24 hours, some nauplii may die normally as their life span is from 24 to 48 hours. The toxicity that was found in the other vials of different concentrations might be due to the toxic property of the plant extracts.[19] *Wrightia arborea Wrightia tinctoria* may contain some cytotoxic constituents that may be soluble in methanol and (70%) ethanol than other solvents used A further research is warranted to investigate the composition and nature of active constituents in these plants leaf extracts.

Acknowledgements

The authors are thankful to Dr. T.K. Ravi, Principal, College of Pharmacy, SRIPMS, Ramakrishna Hospital campus, Coimbatore for his valuable support, in providing the facilities for this study. We are also thankful to Mrs.Amutha Gnana Arasi, Lecturer, Department of Pharmaceutics SRIPMS Coimbatore for her valuable suggestions in carrying out this work.

Table 1. Results of Brine Shrimp Lethality Bioassay for *Wrightia tinctoria*

Drug Group	Sample Conc. (µg/ml)	Log Conc.	No. of Animals Dead n=3			% Lethality for each Group			Mean% lethality/ mortality ± S.D.	LC_{50} (µg/ml)			Mean LC_{50} ± S.D.
70% Ethanol	0	0	0	0	0	0	0	0	0	518.605	454.011	442.197	471.604±41.130[a]
	100	2.000	1	2	2	10	20	20	16.66±5.774				
	200	2.301	3	3	3	30	30	30	30±0.000				
	400	2.602	4	4	5	40	40	50	43.33±5.774				
	500	2.698	5	6	7	60	60	70	60±10				
	1000	3.000	9	10	9	100	100	90	93.33±5.774				
Methanol	0	0	0	0	0	0	0	0	0	562.275	482.741	506.098	517.038±40.880[b]
	100	2.000	1	1	2	10	10	20	13.33±5.774				
	200	2.301	3	2	3	30	20	30	26.66±5.774				
	400	2.602	3	5	4	30	50	40	40±10				
	500	2.698	4	5	5	40	50	50	46.66±5.774				
	1000	3.000	9	9	9	90	100	90	93.33±5.774				

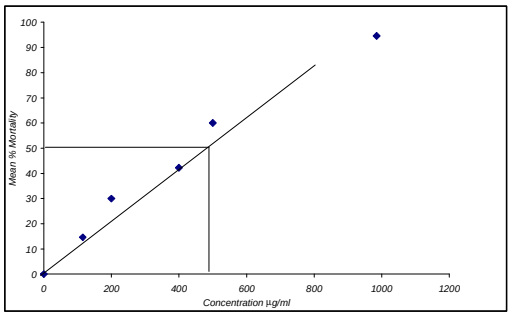
n=5; The results are expressed as mean ±S.D. of three parallel measurements. Values with different superscripts a, b, c, d are not significantly different p value>0.05; whereas a,c & b, d are significantly different p value<0.05. (One way ANOVA done by Dunnett's test.

Table 2. Results of Brine Shrimp Lethality Bioassay for *Wrightia arborea*

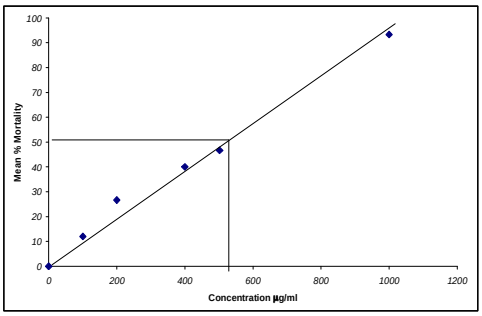
Drug Group	Sample Conc. (µg/ml)	Log Conc.	No. of Animals Dead n=3			% Lethality for each Group			Mean% lethality/ mortality ± S.D.	LC_{50} (µg/ml)			Mean LC_{50} ± S.D.
70% Ethanol	0	0	0	0	0	0	0	0	0	519.792	506.098	468.750	498.213 ± 26.49^{c}
	100	2.000	1	2	2	10	20	20	16.66±5.774				
	200	2.301	2	3	2	20	30	30	23.33±5.774				
	400	2.602	4	4	4	40	40	50	40±0				
	500	2.698	4	5	6	40	50	70	50±10				
	1000	3.000	10	90	10	100	90	90	96.666				
Methanol	0	0	0	0	0	0	0	0	0	500.00	470.370	622.876	531.082 ± 80.865^{d}
	100	2.000	1	2	1	10	20	10	13.33±5.774				
	200	2.301	2	2	2	20	20	20	20±0				
	400	2.602	4	5	3	40	50	30	40±10				
	500	2.698	5	5	4	50	50	40	46.66±5.774				
	1000	3.000	9	10	8	90	10	80	90±10				

n=5; The results are expressed as mean ±S.D. of three parallel measurements. Values with different superscripts a, b, c, d are not significantly different p value>0.05; whereas a,c & b, d are significantly different p value<0.05. (One way ANOVA done by Dunnett's test.

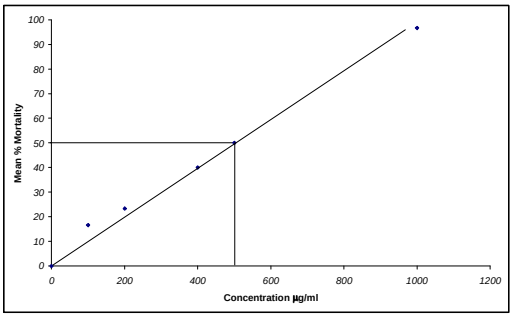
Determination of LC_{50} for *Wrightia tinctoria* 70% ethanolic extract



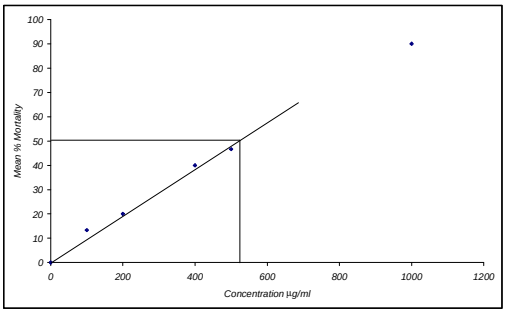
Determination of LC_{50} for *Wrightia tinctoria* methanol extract



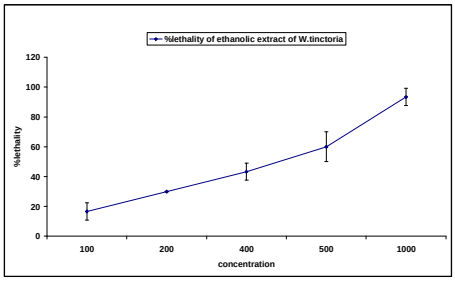
Determination of LC_{50} for *Wrightia arborea* 70% ethanolic extract



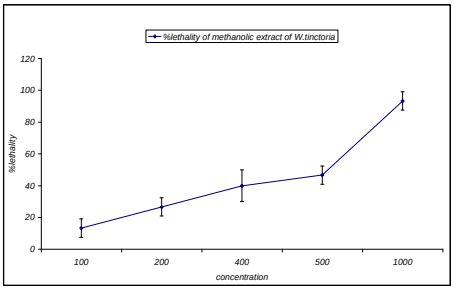
Determination of LC_{50} for *Wrightia arborea* 70% ethanolic extract

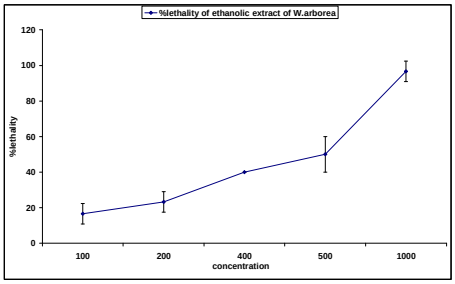


Determination % Lethality of 70% ethanolic extract of *Wrightia tinctoria*

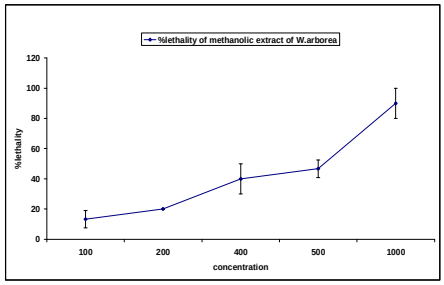


Determination % Lethality of methanolic extract of *Wrightia tinctoria*





Determination % Lethality of 70% ethanolic extract of *Wrightia arborea*



Determination % Lethality of methanolic extract of *Wrightia arborea*

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Anti- Inflammatory Activity of Leaf Extracts of *Alternanthera sessilis*

Subhashini T[1], Krishnaveni B[2] and Srinivas Reddy C[3]*

Department of Pharmacognosy & Phytochemistry, Vaagdevi College of Pharmacy, Warangal -506001, India.

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Abstract

Chloroform and Petroleum ether extracts of leaves of *Alternanthera sessilis* were screened for antinflammatory activity by carrageenan induced rat paw edema method. Both the extracts showed considerable dose dependent activity. However chloroform extract 200mg/kg showed higher activity than other extracts.

Key words: *Alternanthera sessilis Linn*, Amaranthaceae, Carrageenan, Anti-inflammatory activity

1. Introduction

Alternanthera sessilis linn (Amaranthaceae) is an annual or perennial prostate herb with several spreading branches, bearing short petioled simple leaves and small white flowers, found through out the hotter part of India, ascending to an altitude of 1200m[1]. The plant consists of α and β spinasterol[2], lupeal isolated from roots[3]. Plant also contains β- sitosterol, stigmasterol etc[4]. In the indigenous system of medicine the herb has been reported to be used as galactogogue, cholagogue, febrifuge and in indigestion problems[5]. The leaves were used in eye diseases, cuts, wounds and antidote to snake bite; skin diseases [3]. Literature review also indicated that anti-inflammatory property of this species has not been clinically evaluated so far. The present paper reports the anti-inflammatory potency of leaf extracts of *Alternanthera sessilis.*

*For Correspondence: challa_cnu@yahoo.com
Contact: +9198 85108060

2. Experimental

Fresh leaves of *Alternanthera sessilis* were collected from local areas of Warangal, AP, India. The plant was identified and authenticated by S.Vastavya, Associate Professor, Department of Botany, Kakatiya University, Warangal, India. A voucher specimen (As-8-2006) is maintained in Phytochemistry and Pharmacognosy department of Vaagdevi College of Pharmacy, Warangal, India. The leaves were separated, washed, air dried and ground to powder and extracted successively with petroleum ether (60-80^{0}c), chloroform, ethyl acetate, methanol and water by cold maceration. All the extracts were concentrated in vacuum using rotary flash evaporator. The yields were 1, 2.24, 4.36, 9.5 and 10% respectively. Qualitative investigations[6] of petroleum ether and chloroform extracts revealed the presence of steroids, triterpenoids, glycosides, flavonoids and tannins.

Male wistar rats (150-180g) were used to carryout the anti-inflammatory activity. They were maintained under standard environmental conditions and have free access to feed (Nutrient animal feed, Rayan Biotechnology Pvt. Ltd) and water during quarantine period. The institutional animal ethics committee (1047/ac/07/CPCSEA) of Vaagdevi College of Pharmacy, Warangal, A.P, India, approved the animal experimental protocol.

The chloroform and petroleum ether extracts were evaluated for anti-inflammatory activity using carrageenan induced rat paw edema method[7]. The animals were fasted overnight before experimentation, but had been allowed free access to water. Rats were divided into six groups of six animals in each. Group I served as a control and received 1ml/kg of 2% gum acacia orally. Group II served as a standard and received diclofenac sodium 10mg/Kg. Group III to VI received chloroform and petroleum ether extracts at a dose of 100 and 200 mg/kg in 2% gum acacia suspension by oral gastric intubation.

After one hour, edema was induced in all the animals by injecting 0.1ml of freshly prepared 1% carrageenan in normal saline in to the sub plantar region of the right hind paw. The paw volume was measured with Plethysmograph at 0,1,2,3 and 4 hours after carrageenan injection. The percentage of inhibition of edema was calculated using formula:

$$\% \text{ Inhibition of edema} = (1\text{-}Vt/Vc) \times 100 \quad [8, 9]$$

Where Vt = Paw volume in test group animals. Vc = Paw volume in control group animals.

The results were reported as mean $\pm$ S.E.M. The significance of results was calculated using student't' test and was considered statistically significant at $^*P < 0.05$.The results are tabulated in table.1

3. Results and Discussion

The results of Anti-Inflammatory activity revealed that chloroform and petroleum ether extracts exhibited dose dependent activity. At the dose of 200mg/kg the chloroform extract have shown maximum inhibition of the edema (67%) which is comparable to the standard drug diclofenac sodium effect (73%). The detailed results are shown in table.1.

Carrageenan induced paw edema method is a standard and most commonly used technique to screen the acute inflammatory activity[7]. The development of Carrageenan induced inflammation is a biphasic event. First phase occurs with in an hour of injection of phlogistic agent and is mediated through release of histamine, serotonin and kinins while the second phase which can be measured around 3 to 4 hours is related to release of prostaglandins[10].

In the present study chloroform extract showed slight inhibition of inflammation in first phase and maximum inhibition is observed in second phase, which is mainly due to release of prostaglandins. Whereas petroleum ether extract exhibited less effect than chloroform extract. The possible anti-inflammatory effect may be due to inhibition of cyclooxygenase enzyme which catalyzes the biosynthesis of prostaglandins and thromboxane from arachidonic acid. The anti-inflammatory activity of plant sterols has been already established[11, 12]. The Phytochemical investigations revealed the presence of sterols in *Alternanthera sessilis* leaf extract. The present activity may be due to presence of sterols.

Table: 1. Anti-inflammatory activity of *Alternanthera sessilis* on carrageenan induced rat paw edema.

Group/ Treatment	Dose (mg/kg ,p.o)	Mean paw edema (ml)± S.E.M			
		1hr	2hr	3hr	4hr
Group I/ Control	-	0.22± 0.04	0.29±0.05	0.34±0.06	0.27±0.06
Group II/ Diclofenac Sodium	10	0.06±0.02* (72%)	0.13±0.03 (55%)	0.09±0.04* (73%)	0.08±0.03 (70%)
Group III/ Chloroform Extract	100	0.14±0.04 (36%)	0.2±0.02 (31%)	0.17±0.03* (50%)	0.17±0.03 (37%)
Group IV/ Chloroform Extract	200	0.08±0.01* (63%)	0.16±0.02 (44%)	0.11±0.03* (67%)	0.10±0.02 (62%)
Group V Petroleum Ether extract	100	0.17±0.02 (22%)	0.22±0.03 (24%)	0.21±0.04* (38%)	0.2±0.01 (22%)
Group VI Petroleum Ether extract	200	0.15±0.03 (31%)	0.21±0.03 (31%)	0.18±0.04* (47%)	0.19±0.04 (29.6%)

Results are expressed as mean ± S.E.M. (n=6). The significance of results was calculated using student't' test and was considered statistically significant at *P < 0.05

4. Conclusion:

In conclusion, it is clear that anti-inflammatory activity of *Alternanthera sessilis* supports its use given in traditional medicine to reduce inflammation. However, further work should be continued to establish the exact mechanism of action.

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Screening and Quantitation of Phytochemicals and Nutritional Components of the Fruit and Bark of *Helicteres Isora*

Gayathri P [1], Gayathri Devi S [2*], Sivagami Srinivasan[3], Saroja S [4]

1. Department of Biochemistry, Tamil Nadu Agricultural University, Coimbatore-641003
2, 3, 4. Department of Biochemistry, Avinashilingam University, Coimbatore-641043

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Abstract

Helicteres isora is a medium sized tree abundantly found in the hills and forests, well known for its use in traditional medicine. The fruit and bark are said to possess several medicinal values. Hence the present study was designed to screen and quantify selected phytochemicals (polyphenols, tannins, total carotenoids, flavonoids) and proximate principles (carbohydrates, protein, fibre, minerals such as calcium, phosphorus and iron). Results indicate that the fruit contained more amounts of polyphenols (317.7 mg/100g), ascorbic acid (80.0 mg / 100g) and carotenoids (1.7 mg / 100g) than the bark. The bark contained more amounts of tannins (205.1mg / 100g), flavonoids (42.0 mg / 100 g), α-tocopherol (44.0 mg / 100g) and reduced glutathione (184.6 mg / 100g) when compared to the fruit. Among the nutrients, the fruit contained more phosphorus (103.6 mg / 100g) and the bark contained appreciable quantities of total carbohydrates (41.8 mg / 100g), calcium (526.7 mg / 100g) and iron (35.2 mg / 100g) than the fruit. Appreciable quantities of the phytochemicals and nutraceuticals may attribute to the medicinal and nutritive values of *Helicteres isora*.

Key words: *Phytochemical, phenols, tannins, flavonoids, saponins, steroids, alkaloids, calcium, phosphorus, iron, Helicteres isora.*

1. Introduction

Plants with medicinal properties, the gift of Mother Nature to mankind, are in use for centuries in the traditional system of medicine like Ayurveda, Siddha and Unani, in India. Medicinal plants are nature's priceless gift to human beings.

*For Correspondence: devigayathri75@yahoo.co.in
Contact: +91 944 3935039

The herbal medicines are being used by about 80 per cent of the world population for primary health care, particularly in the developing countries[1]. These drugs are popular for its safety and efficacy and are used in the treatment of diseases that have long defied synthetic drugs[2].

Helicteres isora, is a medium sized tree with brick red flowers, belonging to the family of *Sterculiaceae*. It is referred as Modimodika (Oriya), Modaphala (Bhuyan), Valampuri (Malayalam), Valampuri or Tirugupalai (Tamil). Flowers appear in the month of September to December, fruits in the month of January to March. The fruit and bark of the plant are used for treating various diseases and in particular for the treatment of diabetes[3] . The fruit is boiled with mustard oil, filtered and used for massaging legs of patients suffering from gout, twice a day for five days[4]. Extracted juice from the raw fruit of *Helicteres isora* is mixed with equal quantity of mustard oil or ground the fruit along with *Cyanodon dactylon* and mixed with turmeric paste, and is used for massaging the body of children to relive them of profound weakness[5] .

Hence based on the review about the therapeutic values of *Helicteres isora*, the present study was attempted to screen the phytochemicals, quantify them and analyze for selected nutritional components such as carbohydrates, protein, fibre, minerals like calcium, phosphorus and iron.

2. Materials and Methods

2.1 Collection of the fruit and bark

Dried fruits of *Helicteres isora* were obtained from the local market and the bark of the tree was collected from Velliyangiri hills, Coimbatore. The specimen was identified and confirmed by the Botanical Survey of India, Tamil Nadu Agricultural University, Coimbatore.

2.2. Qualitative detection of phytochemicals

Qualitative analysis of the fruit and bark was carried out systematically to identify the phytochemicals. Protein was tested using biuret reaction and xanthoproteic reaction[6] , carbohydrates by Molisch's test[7], tannins by lead acetate test[8], flavonoids by Shinoda test[9], steroids and terpeniods by Salkowski's test and Lieberman-Burchard reation[10], alkaloids using Wagner's reagent[8], phenols using neutral ferric chloride reagent[11] and saponins using sodium bicarbonate[8] .

2.3. Quantification of phytochemicals

Phenols were extracted from the fruit and bark of *H.isora* using 80% ethanol, centrifuged and collected the supernatant. Residue re-extracted, centrifuged, pooled the supernatants and evaporated to dryness. Dissolved residue in distilled water and estimated phenols by the method of Malick and Singh using pyrocatechol as the standard[12]. Tannins were extracted by gently heating the powdered material of the sample with distilled water for 30 minutes, centrifuged at 2000rpm for twenty minutes and collected supernatants. Made up the volume with 100ml of water and estimated by the method of Schanderl [13]. Flavonoids were extracted by grinding the fruit and bark sample, in two steps, firstly with methanol:water (9:1) and secondly with methanol:water (1:1) using sufficient solvent, left for six to twelve hours and filtered to separate the extract. Combined the extracts and evaporated to about 1/3rd the original volume and estimated by the method of Cameron *et al.* [14].

Sample for carotenoids was prepared by extracting the fruit and bark of *H.isora* with petroleum ether repeatedly till the aqueous layer turned colourless and estimated by the method of Zakaria *et al* [15]. Tocopherols were estimated using Emmerie-Engel reaction as described by Rosenberg[16].

The sample was homogenized and allowed to stand overnight with 0.1 N H_2SO_4, filtered and used aliquots of filtrate for the estimation. Reduced glutathione was quantified by the method of Moron *et* al[17]. The sample was homogenized in 5% TCA, precipitated protein was centrifuged at 1000 rpm for ten minutes and supernatant was used for the estimation. The above sample after treatment with a pinch of charcoal was estimated for ascorbic acid by the method of Roe and Kuether[18].

2.4. Quantification of proximate principles

The fruit and bark were quantitatively analyzed for selected nutrients like carbohydrate, protein, fibre, and minerals such as calcium, phosphorus and iron. Total carbohydrates was estimated by the method of Hedge and Hofreiter[19] , protein by the method of Lowry *et* al [20] and fibre content by the method of Raghuramulu *et* al [21]. The ash for the determination of the mineral content was prepared by completely charring the sample over a low flame and heating in a muffle furnace for about three to five hours at 600^{o}C , cooled and obtained the ash. The ash was moistened with distilled water and added distilled HCl, evaporated to dryness on a boiling waterbath (twice), filtered and made up the volume to 100 ml. Calcium was estimated according to the method of Clark and Collip[22], phosphorus by the method of Fiske and Subbarao[23] and iron by the method of Oser[23].

3. Results and Discussion

3.1. Identification of the phytochemicals in the fruit and bark of Helicteres isora

The fruit and bark of Helicteres *isora* was screened qualitatively for the presence of various phytochemicals, the observation and results are depicted in Table I.

3.2. Quantification of phytochemicals

The amount of polyphenols, tannins, flavonoids, carotenoids, tocopherol, reduced glutathione were quantified as per the methods described and the values are expressed as mean ± SD (Table2). It is evident from the results (Table II) that the fruit and bark of *Helicteres isora*are very good sources of polyphenols, tannins, flavonoids and vitamin E than carotenoids and glutathione.

Ascorbic acid plays a central role in pulmonary function, immune response, prevention of coronary heart diseases, cancer and cataract. It also enhances the absorption of non-heme iron from food. It acts as the main radical acceptor from vitaminE itself acts as a scavenger of oxygen radicals and thus inhibits lipid peroxidation[24]. Phenolic compound have been shown to exhibit cellular defense mechanism in atherogenesis and cancer. Polyphenols are a major group of antioxidative compounds, more powerful than vitamin E after it becomes oxidized. They offer protection against LDL oxidation and inhibition of platelet aggregation. A wide array of phenolic substances present in dietary and medicinal plants has been reported to possess powerful antimutagenic activity apart from the antioxidant property.

Recently increasing evidences support the hypothesis that the phenolic compounds could play an essential health promoting role[25]

The pharmacological effects of flavonoids include CNS activity, cardiotonic, lipid lowering, antiulcer, hepatoprotectiv, anti-inflammatory, antineoplastic, antimicrobial antioxidant and hypoglycemic activity. Dietary intake of flavonoids containing foods potentially lowers the risk of certain free radical related pathophysiology[26]. Certain tannins (ellagitannins from *Lagerstroemia speciosa*) stimulate glucose uptake. They exhibit insulin like activity acting as glucose transport activators of fat cells [27]. Number of epidemiological studies has demonstrated that increased intake of natural antioxidants like vitamin A, C, E, flavonoids is very promising in reducing the level of free radicals, severity of diabetic complications, risk of cardiovascular diseases, cancer and many chronic degenerative diseases [28].

Table 1. Phytochemical Screening of the Fruit and Bark of *Helicteres isora*

Phytochemicals	Observation	Inference
Carbohydrate	Reddish violet ring at the junction of two liquids was obtained in Molisch's test	+
Proteins	Violet color obtained in biuret reaction and deep orange color developed in xanthoproteic reaction	+
Polyphenols	Blue color developed with ferric chloride	+
Tannins	White precipitate with lead acetate was obtained	+
Flavonoids	Deep blue color	+
Alkaloids	Yellow brown precipitate	+
Saponins	A honey comb like froth formed	+
Steroids	The upper layer red and the sulphuric layer showed an yellow color with a green fluorescence	+

+ indicates Presence The fruit and bark of *Helicteres isora* contained all the phytochemicals tested.

Table 2. Phytochemical Content in *Helicteres isora*

Compound	Fruit (mg/100g)	Bark (mg/100g)
Polyphenols	317.7 ± 4.2	269.3 ± 2.8
Tannins	180.4 ± 2.3	205.1 ± 1.86
Flavonoids	33.0 ± 0.65	42.0 ± 0.24
Carotenoids	1.7 ± 0.03	1.0 ± 0.01
α – tocopherol	10.6 ± 0.16	44.0 ± 0.94
Reduced glutathione	148.3 ± 1.4	184.6 ± 2.3
Ascorbic acid	80.0 ± 2.3	67.7 ± 3.1

Values are mean ± SD of triplicates

Table 3. Nutritional Components of *Helicteres isora*

Parameter	Fruit	Bark
Total carbohydrates (g/100g)	23.5± 0.25	41.8 ± 0.12
Protein (g/100g)	2.1 ± 0.04	2.3 ± 0.02
Fibre (g/100g)	1.0 ± 0.002	1.5 ± 0.004
Calcium (mg/100g)	45.2 ± 0.10	526.7 ± 4.82
Phosphorus (mg/100g)	103.6 ± 1.2	50.1 ± 1.23
Iron (mg/100g)	23.5 ± 0.34	35.2 ± 0.43

Values are mean ± SD of triplicates

The fruit and bark of *Helicteres isora* are found to contain appreciable quantities of carbohydrate, protein and the essential mineral nutrients necessary for human health maintenance such as calcium, phosphorus and iron. Calcium is important for ossification and iron is necessary for normal hemopoiesis. The bark is found to be rich in all these nutrients except phosphorus compared to the fruit. The fibre content of the fruit and bark of *Helicteres isora* was found to be 1.0 and 1.5 g respectively. Intake of fibre improves the body's handling of glucose and the hormone insulin, perhaps by slowing down the digestion and absorption of carbohydrate. This high level of crude fibre may be helpful in slowing down the carbohydrate absorption and thereby preventing hyperglycemia.

Certain inorganic mineral elements like potassium, zinc, calcium, traces of chromium and magnesium play an important role in the maintenance of normal glucose-tolerance and in the release of insulin from beta cells of islets of langerhans. The fruit and bark are rich in calcium. This may facilitate the efficient release of insulin from beta-cells more efficiently [29]. Increased dietary intake of Ca is currently recommended for the general population to lower the risk of hypertension and osteoporosis. Dietary supplementation of Ca also lowers serum cholesterol [30]. Hence, the fruit and bark may also have hypolipidemic properties due to the good content of calcium.

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Hygeia was the Greek goddess of health and she was worshiped in connection with Aesculapius, her father, the god of medicine and health. She is said to be again, the grand daughter of the God, Apollo. She is also the sister of Panakeia or Panacea (means all-cures) Akeso (Goddess of Healing), and Iaso (which means Remedies). Hygeia is usually depicted as a young woman, who holds a sacred snake (symbolizes resurrection), which is often combined with the rod of Asclepius to form the cadacus, or symbol of medicine. Often this snake is portrayed as drinking from a cup (symbolizing medicine), which has become known as the pharmacist's bowl.

Originally, she was the guardian of physical health and later became the goddess of mental health, as well. Eventually, she became a protectress against various kinds of danger, an attribute which she shared with Aesculapius. It is from Hygeia, the word hygiene originates. Hygiene is the science of preserving health. The subject of hygiene includes all of the agencies affecting the physical and mental well being of people. In its public aspects, it is concerned with soil; climate; character; materials and arrangement of dwellings; heating and ventilation; removal of wastes; medical knowledge on the incidence and prevention of disease; and the disposal of the dead.

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