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Preface

This book covers key areas of Pharmaceutical Research. The contributions by the authors include Kigelia africana, cancer, chemotherapy, leukaemia, chemoprevention, Stem Bark, Fruit and Leaf Extracts, nvention, patent, industrial property, Protection of Inventions, Plant Research, Mucuna pruriens, accessions, L-Dopa, breeding, cultivation, harvesting, Stability, formulation, protein, circular dichroism, Second- Generation Biopharmaceutical Product, Lagenaria vulgaris, Ikshvaku, pharmacognosy, phytochemestry, Hydroxy propyl methy cellulose (HPMC), polymers, swelling, matrices, release rate, Hydrophilic Matrices, 24 h ambulatory blood pressure, blood pressure variability, diabetic nephropathy, glycemic control, glycated hemoglobin, hypertension, Ceiba pentandra, diabetes, liver enzymes, lipid profile, gastric ulcer, indomethacin, Anti-ulcerogenic Effects, Nanotechnology, nanoparticles, solid lipid nanoparticles, nano-emulsion, drug delivery, nanomaterials, Quinoa, gluten-free, germination, glycemic index and glycemic load, New antiretrovirals, HIV-therapy, drug-drug-interactions, Legal highs, synthetic illegal drugs, drug-abuse, drug-drug-interactions, cART, Achyranthes aspera Linn., quantification, oleanolic acid, Thin Layer Chromatography, Oleanolic Acid etc. This book contains various materials suitable for students, researchers and academicians in the field of Pharmaceutical Research.

Chapter 1
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Anti-inflammatory and Anti-leukaemic Activities of the *Kigelia africana* Stem Bark, Fruit and Leaf Extracts

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ABSTRACT

Cancer represents one of the deadliest diseases currently ravaging and killing humans and even animals, and exerts its high array of pathophysiological effects by the unregulated and abnormal cell growth occurring in the different body sites. Leukaemia is a blood or bone marrow cancer that affects haemopoietic stem cell compartment due to somatic mutations in the DNA. Various cancer therapy attempts with the use of chemotherapy and radiotherapy have not demonstrated practical benefits in patients because of their numerous attendant side-effects and cytotoxicity. Several anticancer agents derived from plants are now renowned for their clinical use around the globe because they do not harm normal body cells and *Kigelia africana* plant represents one of such. *Kigelia africana* is an anticancer agent with myriads of beneficial therapeutic properties against microbial infections and cancer cells. Its stem barks, fruits and leaves ethanol extracts possess anti-leukaemic properties and mitigated the associated anaemia of chronic disease and thrombocytopaenia when administered orally to Wister rats following exposure to benzene. Characterization of the various parts of the *Kigelia africana* plant extracts is therefore recommended to determine its bioactive components as a possible promising natural, non-toxic anticancer agent.

Keywords: Kigelia africana; cancer; chemotherapy; leukaemia; chemoprevention.

1. INTRODUCTION

The incidence of deadly diseases globally is alarming, which has geared a number of scientists into research and experiment on various causes of these diseases. One of the deadliest diseases currently ravaging and killing humans and even animals is cancer [1]. Cancer is a broad group of various diseases involving unregulated abnormal cells growth anywhere in the body and develops in almost any organ or tissue and it occurs in many forms of over 200 different types [1]. Leukaemia has been described as a cancer of the blood or bone marrow affecting haemopoietic stem cell compartment, characterised by uncontrolled proliferation and accumulation of malignant leukocytes in the bone marrow and peripheral blood usually results from somatic mutations in the DNA [2]. Some mutations trigger this haematological malignancy by activating oncogenes or deactivating tumour suppressor genes consequently disrupting the regulation of cell death or differentiation. These mutations found even within the African continent and beyond, may occur spontaneously or could result from exposure to radiation or carcinogens such as benzene [3]. Acute leukemias are highly

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malignant neoplasms and are responsible for a large number of haematopoietic cancer-related deaths [4]. Although the survival rates have improved remarkably in the younger age group, the prognosis in older patients is still poor [5,6].

Cancer treatment with the aid of chemotherapy and radiotherapy have not been fully maximized in patients because of their numerous severe side-effects and toxicity, while the use of several anticancer agents derived from plants are being employed in clinical use all around the globe without harming the normal cells of the body [7,8]. Herbal medicines play a vital role in the prevention and treatment of cancer and are commonly available and comparatively economical. Many of these medicinal herbs are presently being investigated for novel drugs or templates for the development of new therapeutic agents [9,10].

Kigelia africana (Lam.) Benth is a tropical African plant found on riverbanks, where it may reach 20m, along streams and on flood plains, also in open woodland, from KwaZulu-Natal to Tanzania [11]. The plant is widely grown and distributed in South, Central and West Africa belonging to the family of *Bignoniaceae* and commonly called the "Sausage tree" because of its huge fruits. The African common names include the ‘pandoro' (Yoruba), ‘uturubien' (Ibo) and ‘Hantsar giiwaa' (Hausa) [12]. It is widely grown as an ornamental plant in tropical regions for its decorative flowers and used throughout Africa, India and the Middle East whereby the tree has been widely cultivated as an endemic species in different habitats for its various medicinal purposes [13,14]. The preparations of *Kigelia africana* have been shown to possess antimicrobial effects [15] and have cytotoxicity against certain cancer cell lines [16,17]. It represents an interesting example of a plant used in traditional medicine for many years, but which is now attracting interest and use far beyond its original geographical range [18,19,20].

Other reported ethno-medical uses of this plant include antioxidant activities [21]. Literature on the chemical composition of *Kigelia africana* via bioactivity-guided fractionation has revealed that the extracts contain various cytotoxic agents, such as lapachol (regarded as a potential anti-cancer drug) [22], norviburtinal [16], kigelinone, and γ-sitosterol [23] among others. This study, therefore, investigated the chemotherapeutic effects of various ethanol extracts of *Kigelia africana* stem bark, fruit and leaf respectively on benzene-induced experimental model of leukaemia in rats using some haematological indices as measuring indicators.

2. MATERIALS AND METHODS

2.1 Plant Material and Extract Preparation

Fresh stem barks, fruits and leaves of *Kigelia africana* were collected from Kajola farm settlement, Ejigbo Local Government Area, Osun State. The plant with the selected parts were identified at the Botany Department, Obafemi Awolowo University and later authenticated at the Department of Pharmacognosy, Obafemi Awolowo University, Ile-Ife, Osun State. The stem bark was oven dried and coarsely powdered, the leaves were air dried and powdered. The fruits were washed, sliced into smaller pieces, oven dried at 40°C and coarsely powdered. 500 g of the plant parts were separately soaked in 1.5 litres of ethanol for 72 hours using the cold soaking/maceration technique and then filtered using Whatman No.1 filter paper. The filtrates were concentrated using rotary evaporator (Stuart Rotary Evaporator model RE:300) with the *Kigelia africana* bark (KAB) yielding 25.2 g (5.04%), *Kigelia africana* fruit (KAF) yielded 40.8 g (8.1%) and *Kigelia africana* leaf (KAL) yielded 30 g (5.8%). They were then stored at refrigerator temperature (2-6°C) until used.

2.2 Animals and Experimental Protocol

Ninety-six Wister strain rats weighing between 150 g and 200 g were purchased from the animal house of Ladoke Akintola University of Technology (LAUTECH), Osogbo Campus, Nigeria and utilized for this research. The rats were randomized into four groups consisting of eight animals per group of two replicates of four for each of the different plant extracts (32 rats per extract of the plant) and subjected to standard 12 hour light/dark cycle. Animals were housed in cages and provided water

and feed *ad libitum*. The rats were allowed to acclimatize for seven days before the commencement of the experiment and the animals' room temperature was maintained at 28±2°C. Rats were randomly grouped according to the treatment received and were examined to be free of wounds, swellings and infections before the commencement of the experiment. All experimental protocols were conducted in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals.

2.3 Administration of Benzene Solution

The Benzene solution used with Cat No 270709 and >99.9% was purchased from sigma Aldrich GmBH (Steinheim, Germany) and diluted in water for injection at a concentration of 1mL of the benzene to 9 mLs of water for injection. Precisely 0.2 mL was administered intravenously through the tail every two days for four weeks. The young rats administered with benzene were allowed to grow under observation and tested periodically until leukaemia developed in significant number of them which were separated for further experimental treatment protocol.

2.4 Acute Toxicity of *Kigelia africana* in Mice

The acute toxicity study of *Kigelia africana* was determined according to the method of Sawadogo and colleagues [24]. Forty eight mice were fasted for 16 hours and randomly divided into eight groups of six animals each. Graded doses (100, 400, 800, 1600, 3200, and 6400 mg/kg) of the ethanolic extract of *Kigelia africana* stem bark, fruit and leaves respectively corresponding to groups II, III, IV, V, VI and VII were separately administered to the mice in each test group by means of an oral cannula. The control group representing group I was administered with distilled water (10 mg/kg) only. All animals were then allowed free access to feed and water and observed for a period of 48 hrs for signs of acute toxicity, morbidity and mortality.

2.5 Administration of the Plant Extract

Ninety-six rats were randomized into groups, consisting of eight animals in each group. Group A; leukaemia control rats were administered benzene (0.2 mL) 48-hourly for four weeks. Groups B received commercial feed with water only, C received Benzene and treatment with ethanol extract of *K. africana* for 3 weeks daily, and D received ethanol extract of *K. africana* plant with commercial diet only (different extracts of *K.africana* stem barks, *K. africana* fruits and *K. africana* leaves) respectively. The ethanol extracts of the various *K. africana* plant was administered by gavage once daily with the aid of oral cannula; the dose administered to the appropriate experimental rats was 0.5 mL of 100 mg/mL stem bark, 0.5 mL fruit and 0.2 mL of the leaf extract for 3 weeks respectively.

Table 1. Experimental protocol

Group	Treatment	Inference
A (n=8)	Leukaemia induction with benzene only	Leukaemia positive control
B (n=8)	Commercial feed with water only	Leukaemia negative control
C (n=8)	Leukaemia induction with Benzene and treatment with Ethanol extract of **K. africana* plant for 3 weeks daily	Chemotherapeutic effect
D (n=8)	Ethanol extract of **K. africana* plant with commercial diet only	Adverse reaction / toxicity

**Ethanol extracts of K. africana stem bark, fruit and leaves were investigated separately using the same protocol in 2 replicates each*

2.6 Sample Preparation

The rats were sacrificed 12 hours after the conclusion of the experiment under light ether anesthesia by cervical dislocation. The blood samples were collected from the inferior vena cava by the use of 5 mL syringe and dispensed into ethylene diamine tetra acetic acid (EDTA) vials, gently mixed and labeled appropriately.

2.7 Determination of Haematological Parameters

Haematological parameters and indices were evaluated by flow cytometry (direct current method) using auto-analyzer Outra O SH800-Plus with the aid of suitable cell packs as described by Akinbo et al. [25]. Differential WBC count was also conducted to complement the automation estimation result using Leishman staining technique as previously described by Bain and Lewis [26].

2.8 Statistical Analysis

Data were expressed as mean ± S.D of two replicates in each group. Analysis of variance (ANOVA) and paired t-test were carried out to test for the level of extract efficacy at $p < 0.05$ among the groups using the statistical package for social sciences (SPSS) 21.0 versions.

3. RESULTS

3.1 LD_{50}/Acute Toxicity Determination

The oral administration of graded doses (100, 400, 800, 1600, 3200 and 6400 mg/kg) of the ethanol extracts of *K. africana* to mice did not produce any significant changes in behaviour, breathing or gastrointestinal effects during the observation period. Also, no deaths were recorded at any point in this study. The LD_{50} of the different parts of *Kigelia africana* is therefore greater than 6400 mg/kg orally in mice.

Haematological parameters such as haematocrit (HCT), total white blood Cell (WBC), red blood cell (RBC), haemoglobin (HGB), platelets (PLT), and red cell indices such as mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) were assessed in the whole blood of experimental rats (Figs. 1-3). Results of the haematological parameters being the major leukaemic indices in the various experimental groups are shown in Table 2. There was marked reduction in the HCT, RBC, HGB and PLT in group A (Leukaemia Positive Control) while the WBC count was markedly increased when compared to other groups indicative of a successful leukaemia development (Figs. 1 and 2).

The RBC indices were significantly ($p<0.05$) increased in the treatment groups than the leukaemia positive control group. *K. africana* treatment of the appropriate groups resulted in significant ($p<0.05$) reduction in the WBC count suggestive of the reversal and alleviation of the leukaemia induction in the treatment group (Table 2).

There were significant ($p<0.05$) differences in all the haematological parameters evaluated in group B (Commercial feed with water only) when compared with A (leukemia induction/positive control) showing significantly elevated leukocyte count and reduced haemoglobin, and packed cells volume in the leukaemia positive group except in platelets and MCV. This confirms that the administered benzene actually induced leukemia in the rats.

Treatment of the healthy rats with all the different ethanol extracts of *K. africana* alongside commercial diet alone (group D) exhibited no significant ($p>0.05$) variation in the evaluated haematological indices when compared with the healthy rats in group B that received commercial feed and water only (Table 2). This revealed that there was no adverse reaction or toxicity experienced by the rats as a result of administration of the various ethanol extracts. The estimated parameters in group B served as reference values.

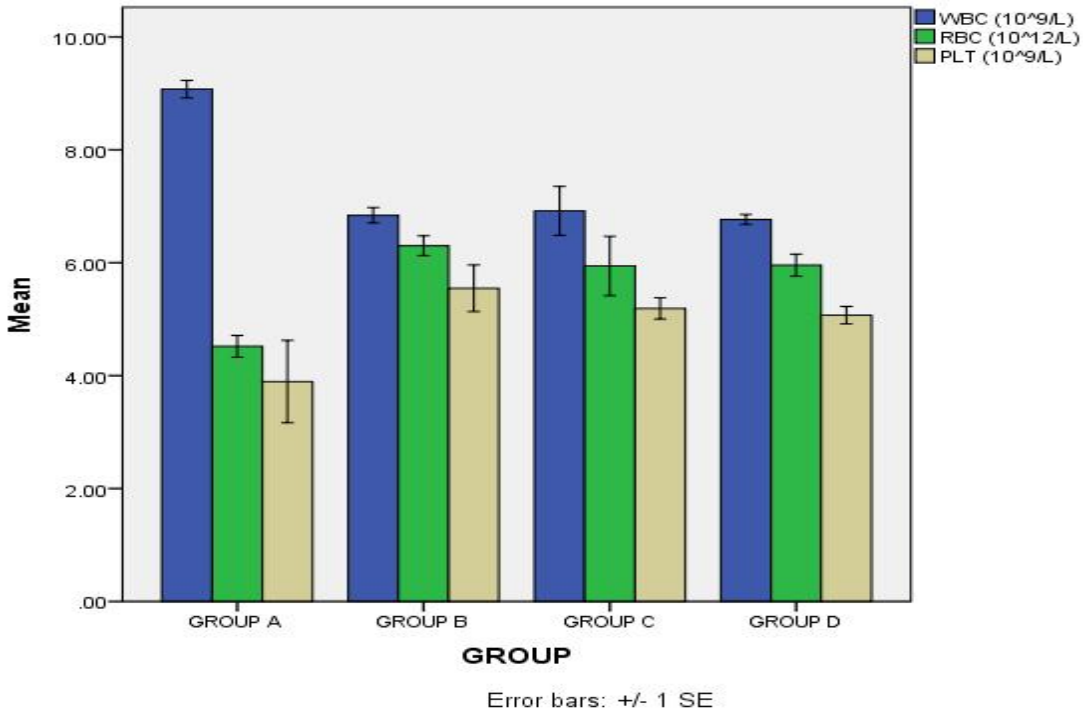

Fig. 1. Some haematological parameters of the respective groups of experimental rats
Bars represent mean ± SEM (n=8). Bars with different statistical markers are significantly different at p <0.05

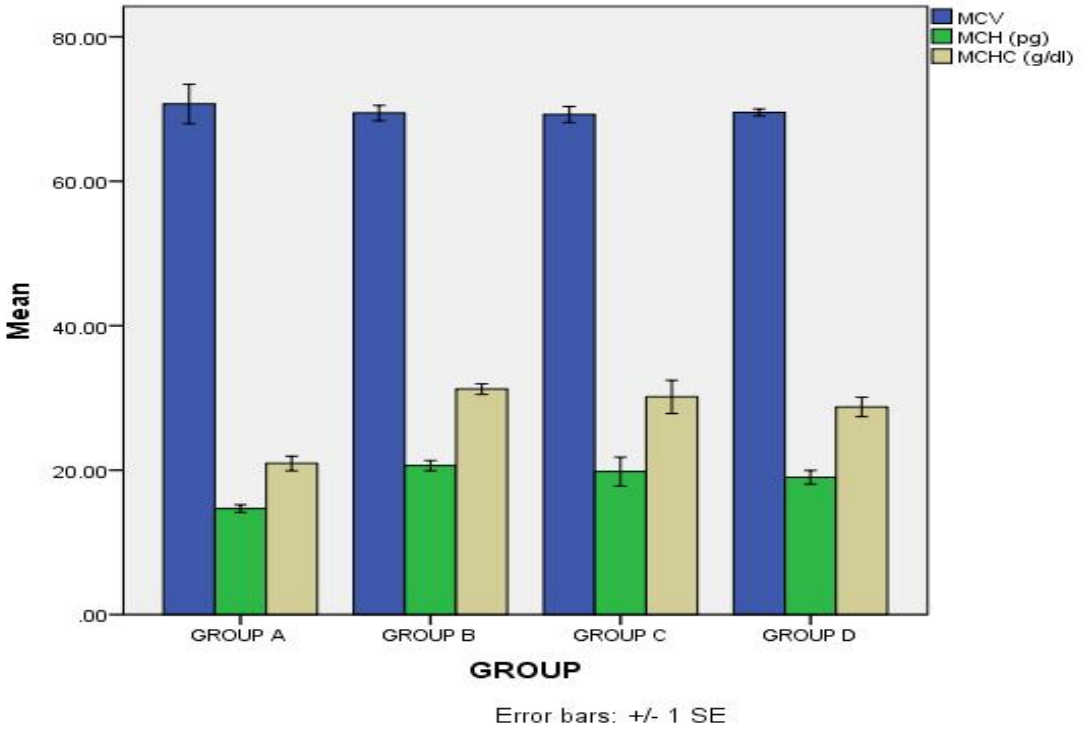

Fig. 2. Typical haematological indices of the respective groups of experimental rats
Bars represent mean ± SEM (n=8). Bars with different statistical markers are significantly different at p <0.05

Table 2. Haematological parameters in the various treatment rat groups using the different extracts of *K. africana* plant

Extract	Group	HCT (%)	WBC (10^9/L)	RBC (10^{12}/L)	HGB (g/dL)	PLT (10^5/µL)	MCV (fl)	MCH (pg)	MCHC (g/dL)
KASB	A	31.88±2.59	11.08±0.31	4.52±0.39	6.68±0.96	3.90±1.46	70.70±5.48	14.67±1.09	20.92±2.03
KASB	B	43.74±1.72*	6.84±0.30*	6.30±0.39*	13.62±0.69*	5.19±0.42	69.24±2.50	20.62±1.56*	31.22±1.64*
KASB	C	41.28±9.48	6.92±0.97*	5.94±1.17	12.90±4.45	5.19±0.42	69.24±2.50	19.80±4.45	30.14±5.15*
KASB	D	41.48±3.78*	6.77±0.22*	5.96±0.48*	12.00±2.26*	5.96±0.48	69.53±1.19	19.02±2.34*	28.75±3.29*
KAF	A	31.88±2.59	11.08±0.31	4.52±0.39	6.68±0.96	3.90±1.46	70.70±5.48	14.67±1.09	20.92±2.03
KAF	B	43.74±1.72*	6.84±0.30*	6.30±0.39*	13.62±0.69*	5.19±0.42	69.24±2.50	20.62±1.56*	31.22±1.64*
KAF	C	39.04±7.37	7.40±1.01	5.57±1.01	9.54±4.07	5.13±1.43	69.24±2.50	19.80±4.45	30.14±5.15*
KAF	D	46.88±6.27*	6.77±0.22*	6.68±0.48*	13.43±3.07*	5.46±1.48	69.53±1.19	19.02±2.34*	28.75±3.29*
KAL	A	31.88±2.59	11.08±0.31	4.52±0.39	6.68±0.96	3.90±1.46	70.70±5.48	14.67±1.09	20.92±2.03
KAL	B	43.74±1.72*	6.84±0.30*	6.30±0.39*	13.62±0.69*	5.19±0.42	69.24±2.50	20.62±1.56*	31.22±1.64*
KAL	C	42.02±1.18	6.70±0.55	5.93±0.56	12.18±2.91	4.71±0.29	71.78±2.92	19.34±2.80	30.14±5.15*
KAL	D	40.12±3.27*	6.98±0.18*	5.70±1.81*	13.78±1.29	4.65±0.97	68.36±4.68	18.84±3.79*	28.75±3.29*

Results are expressed as Mean ± S.D. KASB = Kigelia africana stem bark, KAL = Kigelia africana leaves, KAF = Kigelia africana fruit.
*** Indicates significant difference at p<0.05*

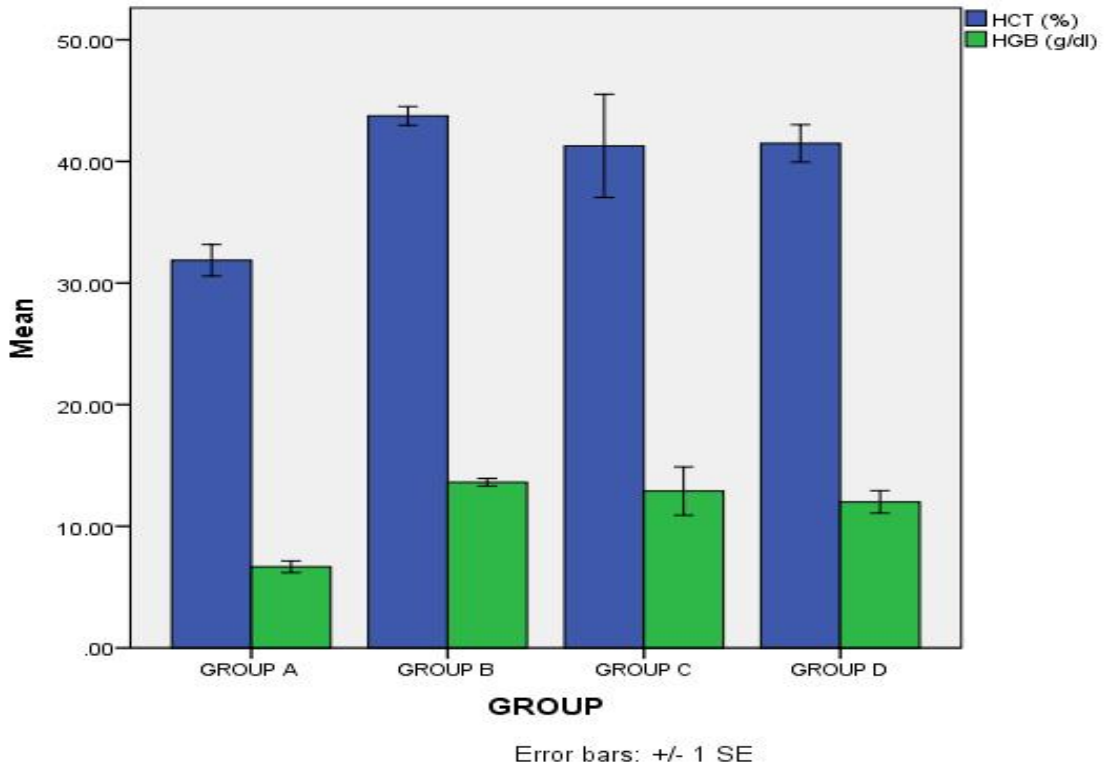

Fig. 3. Haematocrit and haemoglobin levels of the respective groups of experimental rats
Bars represent mean ± SEM (n=8). Bars with different statistical markers are significantly different at p <0.05

4. DISCUSSION

The modulating effect of the different ethanol extract of *K. africana* plant on some basic haematological parameters was analyzed by assessing circulating levels of total WBC counts, PCV/HCT, platelets count, red blood cell counts, haemoglobin and haematological indices in the peripheral blood of control and treatment groups. Anaemia of chronic disease was observed in rat groups where leukaemia was induced due to exposure to benzene solution which is a potent carcinogen. This anaemia is evident by the significant (P<0.05) reduction in HCT, HGB and red cell counts observed in rats in group A when compared with group B (reference value). Anaemia and thrombocytopaenia are considered some of the classical pointers of leukaemia and other haematological malignancies [27]. These established the successful induction of leukaemia in the appropriate groups especially the positive control group. The reversal of these major anaemia indices in the *K. africana* - treated groups suggests that this extract has the ability to enhance the erythropoietic activities in animal models with leukemia and subsequently ameliorating the condition. This study represents an outcome of the *in-vivo* activity of the ethanol extract of *K. africana* stem barks, fruits and leaves.

Benzene-induced rat leukaemia are a suitable experimental animal model for evaluating the anti-leukaemic effect of natural products and are believed to be predominantly mediated via metabolites such as benzene oxide [28]. The central chain of events in the leukaemia-induction concept is based on the idea of marked leukocytosis precipitated by the reactive metabolites of benzene being able to mutate a critical gene or set of genes related to proliferation and differentiation in human stem cells resulting in chromosomal aberrations (aneuploidy, translocations, inversions, and deletions), aberrant mitotic recombination, gene mutations, and/or epigenetic alterations [29]. This was observed in the groups of leukemia-induced rats as reported also in other previous works [30,31]. The leukaemia-positive control groups (benzene treated only) in this study showed significantly higher total WBC

count than the negative control ($P < 0.05$) and treatment groups correlating with the studies listed above. Evidently, the *K. africana*-treated groups showed a significant reduction in the total WBC count evidence of the extract activity. This is consistent with the findings of other previous investigators [16], where they discovered the crude dichloromethane extracts of stem bark and fruit of the plant showed cytotoxic activity *in vitro* against cultured melanoma and other cancer cell lines using the Sulphorhodamine B assay, which was employed for bioassay-guided fractionation. The *in vitro* cytotoxic activity found in root bark extract of *K. africana* has been attributed to a few of its metabolic compounds most commonly γ-sitosterol which is comparable to standard, lapachol [32]. These results substantiate and support the use of the *K. africana* plant extracts as a potent cytotoxic or anticancer agent.

This study showed progressive improvement in the values of HCT, HGB, PLT, MCV, RBC and MCH of the treatment groups when compared to the leukaemia positive control group. This is indicative of the chemotherapeutic activity of the ethanol extract of the plant against leukaemia and is similar to the results obtained in an earlier study where the effect of *Kigelia africana* and 5-Fluorouracil on the body weight of tumor-bearing mice was assessed which showed that the pure compound of *Kigelia africana* exhibited significant anticancer activity when compared with the standard anti-cancerous drug [33,34]. This finding correlates with other previous studies on plant derived anti-leukaemia treatment which revealed the cytotoxicity of ethanol fractions of *Moringa oleifera* on acute myelogenous leukaemia cell culture [35]. The extracts of the plant have been shown to possess various potential anticancer agents [36,37]. The anti-leukaemic activity however appears to be highest in the plant stems bark, and least in the leaf.

Further comparison of the *K. africana* treatment groups with the leukaemia negative control group showed that there were no significant ($P>0.05$) differences in the haematological parameters. Similarly, when rats that were administered with the different ethanol extracts of *K. africana* (group D) were compared with groups fed with commercial feed and water only (group B) there was no significant ($P>0.05$) difference in the haematological parameters. This was an indication of the tolerable nature of the bioactive components of the plant extract on the animals [38,39]. This finding indicates that the ethanol extract of *K. africana* has no toxic effect on the red blood cell parameters i.e. red blood cell count, haemoglobin concentration, packed cell volume, mean corpuscular volume, mean corpuscular haemoglobin concentration and mean corpuscular hemoglobin; also that the administration of *K. africana* plant extract to rats does not produce any deleterious effects on red blood cells and haemoglobin. Therefore, the ethanol extract of *K. africana* has no adverse or toxic effects on healthy haemopoietic cells.

5. CONCLUSION

This study, therefore, shows that ethanol extract of *K. africana* stem barks, fruits and leaves possess anti-leukaemic properties as reflected on benzene-induced leukaemia in Wister rats and thereby mitigating the associated anaemia of chronic disease and thrombocytopaenia when administered orally to rats after exposure to benzene. In the future, phytochemical screening and characterization of the extracts of the various plant parts would help determine its bioactive components as our study suggests that the extract might be a promising natural, non-toxic anticancer agent.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study was approved by the Experimentation Ethics committee on animal use of the College of Medicine, Ladoke Akintola University of Technology (LAUTECH, Reference no. LAU/OSG/380/VOL.XXXI/72). Also, protocols were conducted in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals (1985).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 2
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An Update of the Situation about the Protection of Inventions Derived from Plant Research in a Megadiverse Country: The Case of Mexico

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ABSTRACT

This paper examines the current situation concerning the poor protection of industrial property in Mexico, a country rich in plant species. In addition, plant species have been employed medicinally by indigenous communities and there is also important plant products research being conducted. The protection of industrial property remains an issue of recent introduction in the Mexican economy. The lack of a federal policy regarding science and technology appears to be the main point in increasing patent filing and promoting technology transfer of inventions, as well as protecting of natural resources. We also have discussed possible measures and outlines that could contribute to creating a better situation for involving patenting procedure, especially in the protection of inventions that can be derived from traditional plant knowledge. All suggestions could be useful for placing Mexico in conditions for being a competitive country with the capacity of confronting the present-day challenges of society. Government, industry, research institutions, inventors, and society must actively participate in the creation of a patent culture that allows for protection of the patentable knowledge generated and to provide an impulse for technology, the creation of employment, protection of natural resources, and social and economic rewards, the very basis of a patent: an agreement between inventors and the government to benefit and improve the everyday life of the society.

Keywords: Mexico; invention; patent; industrial property.

1. INTRODUCTION

For many decades in the field of plant products research, many investigations have been focused on developing new alternatives for advancement in providing solutions for unresolved problems, particularly in the health sector, through the generation of medical or pharmaceutical knowledge. Generation of this knowledge comprises the origin of patentable inventions.

Within this context, it can be supposed at first glance that countries with important natural resources and technological development would be able to produce numerous patents that are directed toward solving their own health problems. Even more so, it appears that countries with indigenous communities practicing mainly plant-based medicine could generate patentable inventions due to the crucial role that traditional medicine has demonstrated in access to biological and genetic resources, particularly during the last 15 years. However, some countries with these characteristics have no presence in the industrial property system, especially Latin American countries [1]. This situation can be translated as a great loss, in those economic and social benefits to inventors and indigenous

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communities have not been retrieved, but even worse, the possible enormous benefits on quality of daily life and health are not exerting an impact on society.

The complex mixture of relationships among Research and Technology (R&T), natural resources, indigenous communities, and governments is currently demanding the creation of policies and a solid organization to face the challenges involved in patent protection of plant research-derived inventions. To date, some efforts have been carried out on the international scene; nevertheless, even at the interior of a country —as the case of Mexico— there are differences in the perception and definition of priority problems in patent protection, which combined with certain previous failures, offer a complicated panorama.

2. DEFINITIONS RELATED WITH PATENT PROTECTION

Protection of industrial property (PIP) involves the patent as one of the legal devices that is regulated by the Industrial Property Law in Mexico (LPI) for the protection of inventions derived from technical and scientific knowledge. A patent is an exclusive right of exploitation of an invention that confers the following prerogatives upon its owner: the right to prevent others from manufacturing, using, selling, offering for sale, or importing the patented invention without his/her consent for a period of 20 years. In order to consider it as an invention and as such, susceptible to be protected by a patent, inventions must fulfill the following patentability criteria: *novelty* (meaning anything not in existence in prior art); *inventive step* (the creative process or the results of which are not obviously deducible from prior art by a person skilled in the relevant art), and *industrial application* (the possibility of an invention being produced or used in any branch of economic activity) [2]. The exclusive rights that emanate from the grant of a patent is territorial in nature. As such, an inventor is required to obtain patent protection in all countries in which the invention is to be exploited making use of national registration processes and the right to priority [3]. In many countries and particularly in Mexico, theoretical or scientific principles, essential biological processes for obtaining, reproducing, and propagating plants and animals, biological and genetic material as found in nature, and plant varieties are not patentable according the LPI [4]. The categories of patent protection contemplated by the LPI include products, processes, uses, or apparatuses. Perhaps with the exception of strict studies on systematic taxonomy and ecology, any other area of plant research is able to generate potential knowledge susceptible to protection by patent in all of these categories. Thus, Mexican inventors are presented with the possibility for filing patent applications related to inventions that involve plant research-derived products, processes, and/or uses. According with international laws, all living matter under national maritime jurisdiction have also been declared public property and its exploitation subject to State concessions [5,6].

3. THE CURRENT PANORAMA OF PROTECTION OF PLANT RESEARCH-DERIVED INVENTIONS IN MEXICO

Mexico is considered a megadiverse country in which between 26000 and 30000 plant species thrive, ranking as fourth worldwide, and in first place regarding cactus varieties [7]. Use of Mexican medicinal plants is extensive from preHispanic times and has been widely documented elsewhere [8]. Some studies reveal that traditional Mexican knowledge utilizes approximately 5000 medicinal plants, of which 25% are native from Mexico. Only 16 of these native species were studied by means of a pharmacological approach during the 20th century [9]. Nevertheless, natural products research focused on plants maintains a strong tradition in Mexico; for example, the National Autonomous University of Mexico (UNAM), the most important research public institution in the country, has had specialized research units on natural products for 65 years. Another example is the case of Mexican Institute of Social Security (IMSS), which incorporated a full-time research center dedicated exclusively to Mexican medicinal plants 20 years ago. At present, the IMSS personnel conducts multidisciplinary plant research ranging from traditional knowledge to clinical studies for validating the therapeutic effects of Mexican medicinal plants. Consequently, numerous manuscripts have been published on the plant products research area, especially on native plants. But, had this knowledge been protected by patent?

Between 2012 and 2017 according to the Mexican Institute of Industrial Property (IMPI), a total of 109 940 patent applications were filed, of which 62 182 were granted in Mexico. Although it is calculated that only <3% of these patent applications are filed by Mexican inventors each year, in this period for first time, a significant increasing was registered: 6 643 (approximately 6%) [10]. Mexican applications have been especially focused on areas related with chemistry and metallurgy, diverse industrial techniques, and the food industry [11], but no data are available on the number of patent applications involving plant research-derived products, processes, and/or uses that are filed or granted. Nevertheless, it could be assumed that there are few patent applications related with this technical area, because other reports have shown that 85% of Mexican applications are utility models. According to these data, Mexican inventors are, in practice, not protecting their inventions.

4. INVENTORS, IMPI AND RESEARCH FUNDING

4.1 Researchers Who Become Inventors

One of the most important problems with regard to the PIP in Mexico is that researchers and potential inventors simply are not acquainted with the patenting procedure. There is a total absence of any link to the Industrial Property System during the researchers' academic training. Consequently, they carry out their research activities from undergraduate- to post-doctoral-degree levels disclosing total knowledge generated, which affects possibilities for protecting patentable inventions because in the first instance, these do not fulfill the novelty criteria. Even worse, sometimes these disclosures also affect inventive-step criteria, because some predictions and suggestions for future research are settled in the discussion sections of their own papers. In other cases, in which inventors receive specialized assistance on the patenting procedure from the IMPI, these find that the majority of their investigations do not fulfill requirements for consideration as inventions according to the LPI. After these primary aspects, researchers who possess patentable matter in their investigations begin to deal with the patenting procedure itself; the latter is a legal procedure with very strict deadlines and legal terminology that researchers find difficult to understand and manage. Researchers also consider that the patenting procedure is very risky, because the majority of public research institutions do not recognize and accept patents as an instrument of productivity. Several research institutions employ the publication and citation impact factor count in the Science and Social Sciences Citation Indexes produced by the Institute of Scientific Information (ISI) to monitor and score the performance of their researchers as the base for awarding raises, promotions, and grants. However, these measurements also result in notable limitations, because patents, books, or publications in certain journals do not form part of ISI [12]. Compared with papers in internal productivity evaluations, patents are devaluated; consequently, Mexican researchers prefer to publish in ISI-indexed journals rather than filing patents. Otherwise, researchers who have been interested in filing patents in Mexico have had to suffer from learning the patenting procedure to obtain any type of recognition for their effort, because in many cases, the patent application can be refused in the end. Inventors experience with unpleasant results disappointed with the procedure and results. Moreover, in the case of researchers who were successful in obtaining a patent, these are unaware of how to transfer their technology to industry. In this respect, as patent applicants and technologists, Mexican researchers perceive themselves as completely lost in the Industrial Property System; therefore, patents can represent, from this point of view, a great waste of time.

4.2 The Situation of the IMPI

The IMPI was formally created in 1993 as an autonomous concern [13]. Enormous efforts have been made by the IMPI to promote itself and the patenting procedure. Over the last decade, the IMPI has been imparting free training courses and workshops to train and inform the public on all topics related to the industrial property system and all of the services offered by the IMPI [14]. Additionally, the IMPI has been active in providing consultancy in scientific symposia and to individual research groups, universities, and industry. Thus, many potential inventors have come to begin to know the IMPI and to show interest in the PIP. However, the IMPI remains an institution with the need for consolidation. For instance, since its creation the IMPI has employed an important number of scientific staff as examiners. These in turn left these positions after a brief time because these positions are not recognized efforts. There is a total absence of salary, recruitment, promotion, and training policies, as

well as an excess of pending work that creates professional frustration and low morale [1]. Several permanent and qualified examiners remain a basic necessity of the IMPI to support and offer efficient service. In the field of plants, the IMPI requires examiners trained in industrial property and in ethnobotanical and ethnomedical topics and who are able to evaluate patent applications under an integral perspective [15,16]. In addition, reorganization of the IMPI based on models from other countries appears to be necessary to contribute to create successful institutional policies.

4.3 Research Funding

One crucial aspect that influences low interest in filing patents in Mexico is related with that the majority of public research institutions are not linked with industry, which implicates an absence of financial support for developing new products and filing patent applications. The few research institutions that indeed are linked with industry have dealt with industry that is mainly foreign; one of the conditions for obtaining financial support is to return their results to said industry [17]. Furthermore, industry does not consider research and development in Mexico a top priority nor an attractive long-term investment. Rather, industry tends to acquire a substantial amount of technology based on products or processes from other industrialized countries, therefore limiting the possibilities to create, develop, and innovate high technology and relegating Mexican scientists to the improvement of already existing technology [18].

Precarious Mexican-government investment from R&T over the past 20 years is directly reflected in the low number of Mexican patents, an indicator that not only takes into account creativity, inventiveness, and innovation, but also the capability for cognitive production in industrial applicability, which allows discerning a clear difference between developed economies and advanced countries [19]. Government has not identified areas that are particularly useful to development according to its own needs and resources [17].

Taking together all of this previous data, it is clear that in Mexico, there is no state policy with regard to science and technology sectors. Consequently, Mexico has not been a competitive country, and it possesses an inability to face the challenges of society. Additionally, the protection of industrial property remains a recent introduction in the Mexican economy [13]; therefore, there is no culture related with patent protection. However, Mexico possesses one of the most difficult things to replace: natural resources (especially in plants), and human resources. Some successful isolated cases show that Mexicans have the capacity to increase the protection of their inventions by filing patent applications and contributing to forge better conditions for the population of this country. Other cases in Mexican industry, such as tequila-producing companies, demonstrate that a global technological strategy that involves researchers, industry, and academics results in successful inventions that produce and share benefits at all these levels [20]. Therefore, the missing link existing in Mexico between research institutions and industry is one of the central points to solve for improving the current conditions under which patents and technology transfer represent an enormous and unproductive effort.

5. POSSIBLE MEASURES TO INCREASE THE INDUSTRIAL PROPERTY CULTURE? FUTURE CONSIDERATIONS

There is a vast number of aspects to consider for improving protection of knowledge by patent in Mexico. Mainly, it would appear that informing and about promoting the industrial property interest in terms of potential inventors comprises an indispensable and primary task. For this purpose, the incorporation of basic industrial property-associated concepts into scientific-technological careers requires implementation, from the undergraduate university degree itself to topics related with international treaties and national laws. The writing of a patent application based on experimental thesis work could be validated as thesis work for obtaining a degree, even at postgraduate levels. With these aspects, new researchers could begin to be trained in industrial property, and would be able to direct their own research lines to obtain patentable matter. In this manner, they would be skilled in industrial property issues and could be employed in, for example, patent offices, firms, industry, or universities. These actions can be incentives for scientist to pursue more generalized projects by cooperating not only with other researches from different disciplines, but also with

industrials to obtain diverse funding sources. In this regard, “pure science”, directly related with the most frequently encountered mentality of Mexican researchers, where there prevails a negative and pejorative attitude toward patentability due to the supposed mixing of “pure science” with “business”, may result in a positive patenting attitude, one that permits the emergence of scientist-entrepreneurs [18]. As a positive aspect in this regard, researchers can consider that projects of great importance to Mexico will attract increased interest and promote collaboration with foreign colleagues and industry.

The latter idea is accompanied by the need for research institutions to create a full-time dedicated office in industrial property and transfer technology. One of the most relevant attempts in Mexico has been the creation of the Center for Technological Innovation (CIT) within the UNAM in 1983. At the time, CIT provided several services, which entertained the priority that researchers be involved in all patent and technology-transfer issues in order to sensitize them concerning the field of innovations [21]. However, the CIT was unable to consolidate its presence. With these facts, it is noteworthy that interest in industrial property and transfer technology is on the rise among Mexican researchers.

Traditional knowledge and access to biological and genetic resources comprise another decisive aspect. Certain international efforts have been made with the Trade-related aspects of Intellectual Property Rights (TRIPS) agreement, where standards for the protection of intellectual property rights are pretended to be incorporated into the national legislation of each member country. However, the TRIPS agreement deals with well-specified private rights that do not contemplate an integral form of intellectual property, creating an important gap [22]. On the other hand, the Biological Diversity Convention (BDC) considers principles regarding the conservation and use of biological and genetic resources and defining conditions for the access to these. BDC contemplates sharing benefits among countries, indigenous or local communities, and users in the modern sector, including innovations and practices [15,22,23,24]. Nevertheless, concepts of protection and matter susceptible to be protected continue to be discussed in an international scenario due to the lack of clarity of the objectives and the intrinsic complications of these topics. Thus, actions have been limited, especially in some megadiverse countries [24,25,26]. In this respect, Mexico has no clear position. Perhaps, a first step of inventors in all scientific research disciplines involving plants could become involved in the complex, expensive, and time-consuming task of protecting our natural plant resource by means of offering the integral approaches and patentable results of their investigations, not only as an option, but also as a responsibility [19].

Another critical consideration is the role of the Mexican Government, which through the National Council of Science and Technology (CONACyT) must create a state policy in science and technology that involves not only 1% of the Gross domestic product (GDP) [27], but also an integral strategy that permits to utilize natural resources as a priority for research focused on public health, developmental technology, and conservation. The understanding of the impact of patents on the national economy [19] and attention directed toward the needs of industry linked with research will be key in the formulation of a commitment to invest in and to finance the projects that could generate knowledge and patentable products [1].

Finally, the quality and availability of local resources, government policies, research and technology institutions, industrial investment, and commercial regulations comprise the main aspects to be taken into account in establishing a solid competitive organizational structure that could be of benefit to all the persons involved in the protection of plant research-derived inventions by patent in a megadiverse country such as Mexico. Notwithstanding this, a change of attitude by Mexicans appears basically to be the priority.

6. RECENT ADVANCES IN PROTECTION OF INVENTIONS IN MEXICO

From 2009 to the moment some actions have been afforded in Mexico concerning to protections of inventions that promote the patenting. Especially, an increasing number of patent office created in universities and investigation centers [28]. However, some of these efforts have not been successful results and still are not consolidated. Nonetheless, the general information about protection of inventions and intellectual property currently is definitively information more spreader than one decade ago.

Other interesting advances inside some government institutions such as the Mexican Institute of Social Security (IMSS), has been focused in the incorporation of the patents as productivity marker useful for internal evaluations of researchers and also promoting the patent procedure by a web site [29]; however, once that title of patent have been obtained, no transfer of technology has been occurred yet. Then, at moment, patents represent a great effort for researchers without any retribution.

Concerning the IMPI has been continuously in promoting itself and the patenting procedure. Recently, the web site of the IMPI has been modernized included guides for inventors about the general procedure, cost and how to contact them [30]. Even more, some extracting of the current law about industrial property has been included in the same web. In the beginning of this year, January 2020, Mexico and USA signed an agreement through their patent offices (IMPI and USPTO, respectively), in order to speed up patent procedures in both countries. This agreement was long discussed by economic sectors in terms of that innovations and inventions are recognized as an essential factor in economic development of both countries; consequently, the challenge for institutions is to protect them and shorten the terms for their registry [31].

On the other hand, results interesting that the consideration of Mexico as a megadiverse country, in terms of patenting, is not different from other megadiverse countries, as the case of Brasil, which studies carried on 2019 year, suggested that plant biodiversity are important for analyzing environmental, social, and industrial diagnostics. Although there is not existence of an indicator or index that related the use of vegetal biodiversity to the production of technological innovation in patents, the results of this study in Brasil revealed the need to formulate these indicators due the potential impact in the development of public policies in the areas of technological innovation and the protection of the environment, particularly in megadiverse countries [32].

The topic about promotion of conservation of biodiversity through patents, and especially in megadiverse countries, is an actual concern that some other authors had also considered. The challenge lies in adjusting the schemes for patents and other forms of intellectual property to suit conservation and sustainable-use objectives [33]. However, in the particular case of Mexico, the conservation of biodiversity is not reflecting any link with patenting, probably because the evolution of patenting in Mexico has remained static during decades, in which even the areas of technology remained unchanged [34]. Thus, a great challenge in patenting is still pending in Mexico.

7. CONCLUSION

As we have presented here, Mexico is a country that is rich in natural sources, and especially in plants. No patent-associated culture has limited economic, social, political, and natural conservation benefits. Enormous efforts have been exerted by inventors and research institutions; however, these efforts have been diluted because these are individual efforts, and the majority of these receive no remuneration.

The absence of a policy in science and technology is a great challenge, not only in the plant products research area, but also in Mexican science in general. Government, industry, research institutions, inventors, and society must actively participate in the creation of a patent culture that allows for protection of the patentable knowledge generated and to provide an impulse for technology, the creation of employment, protection of natural resources, and social and economic rewards, the very basis of a patent: an agreement between inventors and the government to benefit and improve the everyday life of the society.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 3
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Medicinal Plant *Mucuna pruriens*: From Farm to Pharma

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ABSTRACT

Mucuna pruriens commonly known as Kaucha in India, is well known for its Neuroprotective and Aphrodisiac property and has been widely used in Indian System of Ayurveda for the preparations of herbal formulations and tonics since ancient time. Seeds of Mucuna have higher level of L- Dopa compare to other legumes and is a promising drug in the treatment of Parkinson's disease- the second most common neurodegenerative disorder. The present report, focus to highlight the information on wild Mucuna germplasm collected from different geographical regions of India, and screened for higher plant growth, seed yield as well as L-Dopa content of seeds. Superior lines were further employed for breeding and 69 F_1hybrids were developed. Three (3) promising selections through back cross lines have been shortlisted for commercial cultivation having characteristics of white coloured seeds, medium - bold in size, weighing 72-114 gm/100 seeds, yielding 424-810 gm seeds/plant and > 4.5% L-Dopa content. Nutritional statuses of selected 3 lines have been tested for carbohydrates, protein, fat and minerals like Calcium, Phosphorus, Iron, Potassium and Sodium. Agronomic practice has been standardized for the cultivation under South Gujarat condition. The maximum economic yield (seed yield) was observed with plant spacing 1×1 meter (1.9 metric tons/hectare) and 1×1.5 meter (1.3 metric tons/hectare) with chemical fertilizer level 150:40:0 kg NPK or bio-fertilizer treatment (T_4) with Biopline+Phosphurt of commercial products plus N:P:K of 100:60:40 kg/hectare and with individual plant support(bamboo poles of 6-8 feet height) system. Harvesting stage was also fixed-up and pods were harvested in semi dried stage and further drying was carried out under shade.

Keywords: Mucuna pruriens; accessions; L-Dopa; breeding; cultivation; harvesting.

1. INTRODUCTION

The therapeutic potential of plants have been explored by the mankind since ancient time, people looked for drugs in nature to treat various diseases [1]. The plant, *M. pruriens*, widely known as "Velvet bean," is a vigorous annual climbing legume originally from southern China and eastern India, where it was at one time widely cultivated as a green vegetable crop**.** All parts of *Mucuna pruriens* possess valuable medicinal properties. Due to the high concentrations of L-dopa (4–7%), velvet bean is a commercial source of this substance, used in the treatment of Parkinson's disease [2,3,4]. In India, seeds of *M. pruriens* are widely used in Ayurvedic preparation and it is a one of the well-known Vrishya (Spermatogenetic activity) drug mentioned in Ayurvedic texts right from Caraka and Sushrutasamhita period. It is also used as a drug for balya (promotes muscle mass and body weight) and advocated to use for vajikarana chikitsa (Aphrodisiac) [5].

The plant of this species grow over semi–arid scrub forests (during and after rainy season) as a twinner receiving support of adjoining trees and shrubs; it gives 2-7 branches and come in flowering after 63-100 days of seed germination and attains maturity at 7-10 months when mature pods are

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picked. Moreover, harvesting of pods becomes a constraint due to the itching effect of trichomes when it comes in contact with skin. Mucuna is cultivated to increase soil fertility, as a green manure of cover crop conservational agriculture system. *M. pruriens* has gained increased global attention in recent times as promising source of protein diet due to presence of 20-30% of protein content in the seed [6]. Mucuna seeds collected from different locations show different botanical features, and environment has no interference in genetic diversities of Mucuna [7]. Approximately 120 species of Mucuna had been reported so far and 130 species according to the Zipcodezoo Data Base. In India, 15 species were identified and reported [8,9].

Keeping in view the importance of *Mucuna* species, there is a need for selection of superior genetic materials for seed yield and L- dopa content as a cash crop. With this objective in focus, an exclusive collection of germplasm was carried out from different geographical regions of India and their populations grown at Zandu Foundation for Health Care's Farm, at Ambach (Village), Vapi, Gujarat, India. Nineteen (19) selected germplasms have been evaluated in details for genetic variability and correlation analysis in terms of yield associated characters and further used for breeding purpose to develop a non- itching variety with high L-dopa content. This was the first attempt to estimate genotypic and phenotypic variation on the large collection of this species and correlation coefficient analysis was also carried out. We believe that the information generated through the present study is useful to the breeders for improving economic traits including the active ingredient.

2. MATERIALS AND METHODS

Nineteen (19) diverse germplasms (accessions) of Velvet bean (*M. pruriens* (L.) DC) were assembled from different regions of India which included 11 lines with non-itching trichomes and rest 8 with itching trichomes on their pods (Fig. 1). For the screening purpose a field trial was laid out with 4 replications in randomized block design in a total field of 468 m^2. The crop was raised at the spacing of 1.0×1.5 meter. The seeds were sown on the slop of ridges for evaluation. All the plants in the field trial were given supporting stakes of 1.8-2.4 meter poles (Fig. 2). Fifteen yield contributing characters were recorded and subjected to genotypic and phenotypic characterization. Flowering, pod development and seed setting are considered as important parameters for higher yield, so a set of characters have been chosen to evaluate the morphology of flower, seeds, as well as harvesting time of pods. L-dopa content in seed was measured by using standard method [10].

(i) (ii)

Fig. 1. (i) *M. pruriens* accessions- Mature and green pods of different non-itching and itching line; (ii) *M. pruriens* seeds of itching and non itching accessions

(i) (ii)

Fig. 2. (i) *M. pruriens* cultivation field at 1 month crop age; (ii) *M. pruriens* cultivation field at 4 months crop age

The crop was not given irrigation in the first four months (June to September) due to monsoon rains in this region of South Gujarat, India and later surface irrigations (ranging from 3 to 4 times) were given as required during the later period of dry season. The pods with mature seeds were collected at semi -dried stage when they turn brownish- black. The pods were further dried under shade in order to bring down the moisture level of seeds to 10%. These lines were used as a parental line in breeding programme which generated 69 F_1 Hybrids and their BC_1 lines. All were subjected to evaluation for yield; seed associated characters and L- dopa content. The seeds were grouped into three categories based on weight. Heterosis was calculated according to the common method as increase or decrease in the mean F_1 performance over the mid parent, better parent and best parent.

Two selected progenies of *M. pruriens* hybrid lines have been short listed from 69 lines for commercial cultivation under South Gujarat conditions. Both these lines have white coloured seeds, medium bold in size weighing 86-103 gm/100 seeds, yielding 491-532 gm (seed)/plant containing 5.5-6.0% of L-dopa content. Agronomic practice including effect of plant spacing, NPK fertilizes and various support/stakes on seed yield were standardized. Four different spacing (0.5×0.5m, 1.0×0.5m, 1.0×1.0m and 1.0×1.5m) were applied to standardize the optimum distance between plants. Twelve combinations of NPK fertilizers have been studied. The following commercial grade commercial fertilizers were used in different combinations.

T_1= Control (10t /ha.,Farm Yard Manure - FYM only); T_2=Control (Viz. 100:60:40 kg/ha N:P:K); T_3= Biopline+Phosphurt (Commercial Biofertilizers Product, No chemical fertilizers); T_4= Biopline+ Phosphurt+N:P:K at 100:60:40 kg/ha full dose); T_5= Biopline+Phosphurt + 75:40:30 kg/ha N:P:K; T_6= Biopline+Phosphurt + 50:30:20 kg/ha N:P:K; T_7= Phosphohurt + 100:60:40 kg/ha N:P:K; T_8=Phosphohurt +75:40:30/ kg/ha N:P:K; T_9= Phosphohurt +50:30:30/ kg/ha N:P:K.

Four stages of developing pods viz. Green (early mature), Turning yellow (Mid-mature), Semi- drying and fully drying have been assessed for seed associated characters and seed quality. Furthermore the effect of various biofertilizers was also studied during optimization of the harvesting stage.

3. RESULTS AND DISCUSSION

The genetic variability studies on yield associated characters presented in Table 1 have revealed a wide range of variation for all the traits in the germplasm studied. Among these, 11 germplasms showed non-itching trichomes and 8 germplasms possessed itching trichomes on their pods largely bearing white, black or striped of greenish brown or gray seed colour (Fig. 3). It was observed that the non-itching trichome bearing germplasm produce early flowering (<107 days) over the rest (Fig. 4). The early flowering is an important yield contributing character and is confirmed over research carried on allied crop called Sword bean (*C. gladiata*) growing in India [11].

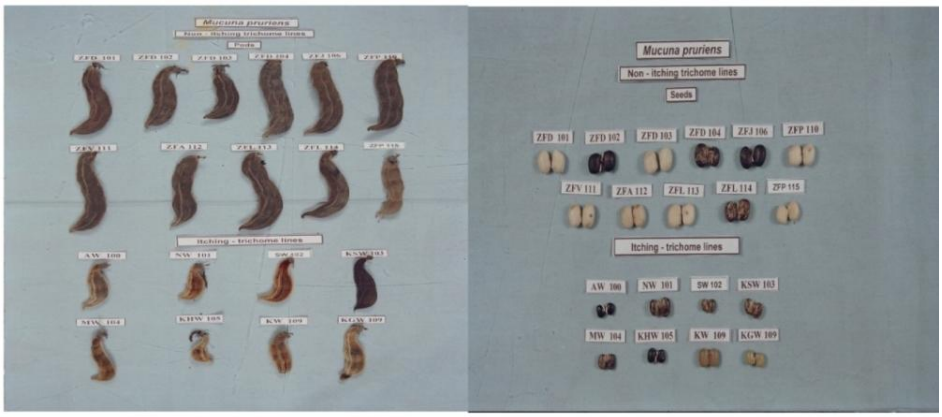

Fig. 3. (i) Dry pods of different non-itching and itching lines; (ii) Seeds of these accessions

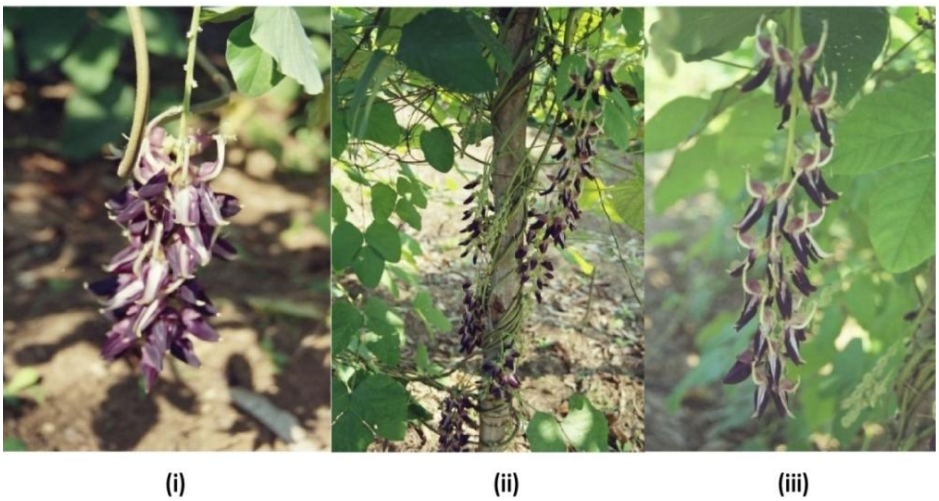

Fig. 4. *M. pruriens* inflorescence (i-iii) at different stages

The mean value of characters (Table 1) showed that these non-itching germplasm (Coded ZFD 101, ZFD 102, ZFD 104, ZFP 110, ZFA 112, ZFL 114 and KSW 115) registered maximum values recorded for length of pod (9.12-15.36 cm), Seed weight/pod (2.35-4.77g), size of cotyledon leaf (49.15-74.70 cm^2), fresh weight of cotyledon (1.00-1.81g) and seed yield (63.53-270.86 g/plant) over predominantly white of black or striped colour seeds as compared to itching trichome germplasm. The itching trichome germplasm generally has blackish-striped or greenish–brown or grey colour seeds. Amongst these, a germplasm namely KW 109 gave higher seed yield (152.55 g/plant) combined with other superior trait of number of flower/plant and 100 seed weight as compared to other itching trichome germplasm. Similarly, the six itching germplasm had exhibited higher values of number of flower/plant, number of pods/plant and days for initiation of flowering over non itching germplasm. In the previous study, a similar observation was confirmed that the cultivated crop of three germplasm of *M. pruriens* collected in India and Stuttgart, Germany, particularly related to physicochemical properties and chemical composition [12]. The occurrence of such a large genetic variability, in natural plant population laid off open avenue for selection of superior lines and further improving their yield traits through plant breeding programme.

Estimates of range, phenotypic and coefficient variance heritability, for the 15 traits among the material studied are shown in Table 2. Low magnitude (0.00-3.83%) of difference between PCV and

GCV for length of pod, length of flower internode, number of pods/inflorescence, weight of seed/pod, 100 seeds weight, initiation of flowering (days), size of cotyledon leaf, fresh weight cotyledon and seed yield/plant indicates relatively low influence of environment and therefore, gave reliability of selection based on phenotypic performance. Of the 19 lines studied for 15 characters, the estimate of heritability (85.0-98.40%) indicates that these characters can be effectively employed for selection of most suitable parent material in a breeding programme. In a similar study, Robinson et al., [13] has used PCV and GCV in selection of superior lines in corn. In the present study, the small difference between PCV and GCV for many characters indicated that this variation occurs due to the additive genes which could be fixed through selection. The high hereditability combined with moderate-high genetic advance (0.98-151.12) observed for some characters such as length of pod, length of inflorescence and flower internodes, number of pods per inflorescence, weight of seeds/pod, 100 seed weight, size and weight of cotyledon and seed yield could be contributed by non-additive gene action and these characters can be further improved by hybridization programme. Similar results have been reported in many tree species of seed/fruits e.g. *Prosopis* species [14,15], *D. sissoo* [16], Walnut [17], *T. chebula* [18]. Johnson et al., [19] demonstrated in soya bean that heritability estimates in conjugation with genetic advance are usually more helpful in predicting the resultant effect of selecting individuals. Similarly, high heritability for number of pods, 100 seed weight and other yield associated characters were observed in *V. sinensis* (cow pea) by several investigators [20,21].

Generally, the magnitude of genotypic correlation co-efficient was found to be higher than the corresponding phenotypic correlation coefficient for seed yield/plant with other parameters studied. It leads us to infer that even though there is a strong inherent association between various characters; the phenotypic expression of the correlation was lessened under the influence of environment (Table 3). This is evident that seed yield/plant has higher corresponding phenotypic correlation coefficient for seed yield/plant with other parameters studied. It also leads us to infer that even though there is a strong inherent association between various characters; the phenotypic expression of the correlation was narrowed under the influence of environment (Table 3). This is evident that seed yield/plant has higher genotypic positive correlation with 100seed weight (0.916), weight of seed/pod (0.896), size of cotyledon (0.916), length of pod (0.871) and fresh weight of cotyledon (0.846). However, the seed yield/plant has lower magnitude of phenotypic positive correlation with weight of seed/pod (0.816**), 100 seed weight (0.805**), length of pod (0.805**), fresh weight of cotyledon (0.771**), size of cotyledon (0.769**) and length of inflorescence (0.738**). Moreover, the seed yield/plant showed higher negative genotypic correlation with length of inflorescence (-0.813), number of flowers /inflorescence (-0.707), number of flowers/plant (-0.494), number of pods/ inflorescence (-0.636), and initiation of flowering (-0.457), which indicates that breeders may opt in selecting those lines which possess short length of inflorescence and lesser number of pods/plant combine with larger number and heavier weight of the seed/pod for improving the seed yield. Furthermore, the size and weight of cotyledon have to be taken as additional indicators for selecting high yielding lines.

The direct and indirect correlation analysis for 15 characters in germplasm of *M. pruriens* showed high and direct positive correlation between seed yield and weight of seed/pod (1.359), which resulted in high total correlation with seed yield (0.896**). Total correlation with seed yield with various characters viz. 100 seed weight, weight of seed/pod, size of cotyledon leaf, length of pod and fresh weight of cotyledon was positive and high (Table 4). The high magnitude of total positive correlation (0.896) for weight of seed/pod is reflected through direct effect (1.359), however, the high total positive correlation with 100 seeds weight (0.916) registered directly via weight of seed/pod (1.28), length of pod (0.115), number of flower per inflorescence (0.197), number of pods/ inflorescence (0.062), initiation of flowering (0.057), percent of pod setting (0.048) and number of flower/plant (0.034). similarly, the high total positive correlation recorded for length of pod, size of cotyledon were mainly the results of indirect effect of seed yield with weight of seed/pod, combined with low magnitude of direct and indirect effect of seed yield with length of pod, number of flowers/inflorescence, number of pods/inflorescence and initiation of flowering (Fig. 4). [22]. Similar findings related to direct and indirect correlation for various characters were made in other legume crop plants namely *V. sinensis* [23] and some tree species like *P. roxburghii* [24], *B. variegata* [25] and *Leucaena* species [26].

Table 1. Characterization of 19 Mucuna accessions for yield associated parameters

Sr. No.	Characters	Germplasm code																	
		ZND 101	ZND102	ZND103	ZND104	ZFJ106	ZFP110	ZFV111	ZFA112	ZFL113	ZFL114	ZFP115	AW100	NW101	SW102	KSW103	MW104	KHW105	KW109	KGW109
1.	Length of inflorescence (cm)	5.56	5.09	4.65	5.42	8.42	13.23.	4.50	4.80	11.29	8.64	31.52	27.12	20.37	24.97	27.80	27.87	16.75	24.72	11.42
2.	Length of pod (cm)	11.10	13.09	12.23	11.76	11.32	11.75	12.20	11.39	13.95	15.36	9.12	8.52	7.02	8.32	7.02	9.27	9.64	8.04	9.67
3.	Length of flower inter node (cm)	0.70	0.79	0.46	0.68	1.71	1.67	0.59	1.83	1.58	1.14	1.17	1.96	1.57	1.07	1.76	1.39	1.27	1.77	0.70
4.	No. of inflorescence/plant	27.71	20.12	21.84	18.00	13.69	19.14	23.18	24.63	18.44	32.95	7.80	24.76	20.80	15.32	25.03	19.80	21.63	20.68	32.50
5.	No. of flowers/plant	222.31	174.98	199.79	161.71	190.69	215.02	165.52	179.35	265.66	339.99	162.66	371.07	322.58	272.29	355.42	322.85	292.63	402.47	352.75
6.	No. of flowers/ Inflorescence	8.03	8.76	9.01	8.96	14.12	11.24	28.63	7.34	14.30	10.46	21.41	14.38	15.46	17.74	14.90	16.19	13.53	13.19	10.91
7.	No. of pods/ plant	97.41	81.47	88.25	58.96	53.84	82.53	78.98	87.21	69.59	81.51	66.86	120.50	115.09	0.41	137.62	104.00	117.31	93.26	151.00
8.	No. of pods per inflorescence	3.38	4.09	4.06	3.27	3.91	4.31	.43	3.54	3.77	4.45	8.58	4.87	5.49	4.71	6.04	5.25	5.45	4.46	4.46
9.	Pod setting (%) from flower/ plant	43.06	46.73	45.68	36.56	28.19	38.39	48.82	50.90	26.59	23.78	41.46	33.61	35.79	28.39	38.05	32.43	40.66	32.46	42.49
10.	Weight of seed/pod (gm)	3.28	3.04	2.97	3.33	4.77	3.74	3.20	2.82	4.19	3.90	2.35	0.98	1.06	0.89	1.02	1.25	1.02	1.16	1.52
11.	100 seeds weight (g)	1.03.34	161.96	84.03	174.62	20.6.03	108.08	107.27	108.26	112.82	119.06	32.53	30.01	37.00	24.16	44.12	29.00	26.97	74.86	28.56
12.	Initiation of flowering (Days)	81.50	74.25	85.50	74.25	117.25	100.50	94.00	92.50	102.25	88.25	78.00	121.00	125.50	90.00	122.50	99.25	108.50	112.50	97.75
13.	Size of cotyledon leaf (cm^2)	55.57	68.25	71.55	62.10	68.37	74.70	63.85	66.82	63.72	62.75	49.15	17.32	25.85	25.15	18.52	23.45	18.45	22.27	27.35
14.	Fresh weight of cotyledon (g)	1.72	1.74	1.62	1.13	1.36	1.03	1.20	1.81	1.21	1.79	1.00	0.37	0.43	0.32	0.26	0.58	0.45	0.27	0.43
15.	Seed yield (g/plant)	174.98 (-,W)	270.86 (-,B)	175.82 (-, W)	187.94 (-,B)	264.50 (-,S)	190.16 (-,W)	263.08 (-,W)	208.24 (-,W)	233.14 (-,W)	245.88 (-,S)	63.53 (-,W)	72.86 (+, S)	64.18 (+,S)	91.22 (+,S)	93.26 (+,S)	93.66 (+,S)	110.54 (+,S)	152.55 (+G, Br)	73.32 (+,G)

[All the figures are the means of four replications; - and + indicates germplasm possessing no-itching and itching and itching trichomes on pods respectively; Seed colour -W: white; B: black; S: striped; G. Br: greenish brown; G: grey] (Source: Krishnamurthy et al., [22])

Table 2. Estimation of genetic parameters

Characters	Range	Mean	Standard error	C.D at 5%	G.C.V (%)	P.C.V (%)	Broad sense Heritability (%)	Genetic advance (G.A)	G.A. as % of mean
Length of Inflorescence (cm)	4.5-31.52	14.992	1.13	2.22	64.31	65.19	97.30	19.59	128.14
Length of pod (cm)	7.02-15.06	10.514	0.33	0.65	21.89	22.34	96.00	4.64	43.27
Length of flower internode (cm)	0.45-1.96	1.255	0.05	0.09	38.62	39.11	97.80	0.98	77.21
No of inflorescence/ plant	7.80-32.95	21.476	2.75	5.39	26.33	32.17	67.00	9.53	43.53
No. of flower/ plant	161.71-402.47	254.856	44.41	87.06	26.43	36.17	53.40	101.42	39.02
No of flowers/ inflorescence	7.34-21.41	12.489	1.66	3.26	29.42	35.04	70.50	6.35	49.99
No of pods /plant	53.84-151	92.263	12.92	25.32	26.60	33.29	64.00	40.44	42.98
No of pods/ Inflorescence	2.45-8.58	4.51	0.34	0.67	29.06	30.97	88.00	2.52	54.98
Pod setting (%) from flower/plant	23.78-50.9	37.63	4.07	7.98	19.09	24.88	58.90	11.36	29.60
Weight of seed/ pod (g)	0.89-4.77	2.449	0.15	0.30	52.10	52.87	97.10	2.59	103.71
100 seeds weight (g)	24.16-206.03	84.877	6.15	12.06	45.62	47.15	93.60	68.21	89.15
Initiation of flowering (Days)	74.25-125.5	98.658	6.85	13.43	16.40	19.12	73.60	28.60	28.43
Size of cotyledon leaf (cm^2)	18.45-47.4	46.591	3.19	6.25	47.12	48.11	95.90	44.28	93.32
Fresh weight of cotyledon (g)	0.27-1.81	0.987	0.05	0.10	1.24	1.24	98.40	1.17	117.22
Seed yield (g/plant)	63.53-270.86	157.984	23.62	46.25	39.22	42.55	85.00	151.12	73.05

[C.D.: Critical difference; G.C.V.: Genotypic coefficient of variation; P.C.V.: Phenotypic coefficient of variation] (Source: Krishnamurthy et al., [22])

Table 3. Genotypic and phenotypic correlation coefficients analysis

Characters	Length of Inflorescence (cm)	Length of Pod (cm)	Length of flower inter node (cm)	No of inflorescence/ plant	No. of flower/ plant	No of flowers/ inflorescence	No of pods /plant	No of pods/ Inflorescence	Pod setting (%) from flower/plant	Weight of seed/ pod (g)	100 seeds weight (g)	Initiation of flowering (Days)	Size of cotyledon leaf (cm^2)	Fresh weight of cotyledon (g)	Seed yield (g/plant)
Length of Inflorescence (cm)	-	-0.79	0.51	-0.36	0.57	0.92	0.335	0.794	-0.385	-0.718	-0.79	0.487	0.774	-0.784	-0.813
Length of pod (cm)	0.758**	-	-0.38	0.195	-0.501	-0.63	-0.576	-0.686	0.016	0.686	0.92	-0.589	0.853	0.84	0.871
Length of flower internode (cm)	0.469*	-0.368	-	-0.161	0.420	0.453	0.143	0.235	-0.425	-0.195	-0.350	0.772	-0.323	-0.364	-0.311
No of inflorescence/ plant	-0.28	0.167	-0.126	-	0.496	-0.633	0.590	-0.540	0.146	-0.076	0.006	0.072	-0.118	0.109	0.306
No. of flower/ plant	0.438	-0.327	0.288	0.563*	-	0.298	0.74	0.135	-0.464	-0.649	-0.72	0.688	-0.829	-0.692	-0.494
No of flowers/ inflorescence	0.783**	-0.497*	0.357	-0.470*	0.398	-	0.01	0.284	-0.483	-0.465	-0.601	0.355	-0.575	-0.644	-0.707
No of pods /plant	0.279	0.438*	0.113	0.647*	0.763**	0.072	-	0.254	0.228	-0.716	-0.68	0.517	-0.730	-0.588	-0.393
No of pods/ Inflorescence	0.738	-0.627**	0.219	-0.463*	0.124	0.725**.	0.267	-	0.086	-0.55	-0.569	0.211	-0.507	-0.548	-0.636
Pod setting (%) from flower/plant	-0.303	-0.004	-0.299	0.080	-0.461*	-0.539	0.170	0.112	-	-0.046	0.158	-0.340	0.245	0.301	0.194
Weight of seed/ pod (g)	-0.699**	0.836**	-0.199	-0.096	-0.501*	-0.387	-0.610	-0.519*	-0.064	-	0.942	-0.432	0.932	0.822	0.896
100 seeds weight (g)	-0.754**	0.877**	-0.339	0.025	-0.515*	-0.511	-0.519	-0.525*	0.127	0.899**	-	-0.621	0.966	0.913	0.916
Initiation of flowering (Days)	0.414	-0.525*	0.660*	0.103	0.501*	0.298	0.382	0.147	-0.333	0.364	-0.522*	-	-0.606	-0.664	-0.457
Size of cotyledon leaf (cm^2)	0.751**	0.811**	-0.310	-0.104	-0.609*	-0.485*	-0.591*	-0.481*	0.176	0.897**	0.931**	-0.499*	-	0.898	0.882
Fresh weight of cotyledon (g)	-0.769**	0.822**	-0.357	0.086	-0.501*	-0.538*	-0.472*	-0.514*	0.219	0.804**	0.882**	-0.575*	0.867**	-	0.846
Seed yield (g/plant)	0.738**	0.805**	-0.278	0.338	-0.201	-0.523*	-0.136	-0.503*	0.149	0.816**	0.805**	-0.432	0.769**	0.771**	*

*[** Significant at 1% levels of significance; * significant at 5 % levels of significance] (Source: Krishnamurthy et al., [22])*

Table 4. Estimate of direct and indirect of seed yield with related yield associated characters

Characters	Length of Inflorescence (cm)	Length of Pod (cm)	Length of flower internode (cm)	No of inflorescence/ plant	No. of flower/ plant	No of flowers/ inflorescence	No of pods /plant	No of pods/ Inflorescence	Pod setting (%) from flower/plant	Weight of seed/ pod (g)	100 seeds weight (g)	Initiation of flowering (Days)	Size of cotyledon leaf (cm^2)	Fresh weight of cotyledon (g)	Total correlation with seed yield
Length of Inflorescence (cm)	1	-0.098	-0.02	-0.108	-0.03	-0.301	0.046	-0.086	-0.12	-0.975	0.082	-0.045	-0.014	0.108	-0.81**
Length of pod (cm)	-1	0.125	0.014	0.059	0.024	0.206	-0.079	0.074	0.005	1.169	-0.095	0.054	0.015	-0.166	0.871**
Length of flower inter node (cm)	0	-0.047	-0.04	-0.049	-0.02	-0.148	0.02	-0.026	-0.13	-0.265	0.036	-0.006	0.05	0.05	-0.31
No of inflorescence/ plant	0	0.024	0.006	0.303	-0.02	0.207	0.081	0.059	0.042	-0.103	-0.0001	-0.0007	-0.0002	-0.015	0.206
No. of flower/ plant	0	-0.063	-0.02	0.15	-0.05	-0.098	0.102	-0.015	-0.14	-0.882	0.075	-0.062	-0.015	0.096	-0.49*
No of flowers/ inflorescence	1	-0.079	-0.02	-0.192	-0.01	-0.328	0.0001	-0.089	-0.15	-0.632	0.062	-0.33	-0.01	0.089	-0.71**
No of pods /plant	0	-0.072	-0.01	0.179	-0.04	-0.003	0.137	-0.028	0.068	-0.973	0.071	-0.048	-0.013	0.081	-0.39
No of pods/ Inflorescence	1	-0.086	-0.01	-0.163	-0.01	-0.27	0.035	-0.108	0.026	-0.748	0.059	-0.019	-0.008	-0.076	-0.64*
Pod setting (%) from flower/plant	0	0.0002	0.016	0.042	0.022	0.158	0.031	-0.0009	0.301	-0.062	-0.016	0.031	0.0004	-0.042	0.194
Weight of seed/ pod (g)	-1	0.107	0.007	-0.023	0.031	0.153	-0.098	0.06	-0.01	1.359	-0.097	0.04	0.016	-0.144	0.896**
100 seeds weight (g)	-1	0.115	0.013	0.0002	0.034	0.197	-0.093	0062	0.048	1.28	-0.103	0.057	0.017	-0.126	0.916**
Initiation of flowering (Days)	0	-0.074	-0.03	0.022	-0.03	-0.166	0.071	-0.023	-0.1	-0.587	0.064	-0.092	-0.011	0.092	-0.46*
Size of cotyledon leaf (cm^2)	-1	0.106	0.012	-0.036	0.039	0.189	-0.1	0.55	0.074	1.266	-0.1	0.0556	0.018	-0.124	0.882**
Fresh weight of cotyledon (g)	-1	0.105	0.014	0.033	0.033	0.211	-0.08	0.059	0.09	1.117	-0.094	0.061	0.016	-0.138	0.846**

(Source: Krishnamurthy et al., [22])

The path coefficient analysis study indicated that length of pod, weight of seed/pod, number of flowers/inflorescence, percent of pod setting, 100 seed weight, size of cotyledon, weight of cotyledon and early flowering exerted the greatest direct and indirect influences upon overall seed yield and these are the stable heritable characters which could be used in selecting higher seed yielding germplasm in a breeding programme. Out of the 19 collected lines, 17 lines with superior characteristics were used in breeding programme (Table 5).

Table 5. Seed associated characteristics of 17 Mucuna germplasm

Sr. no.	Parents Code	Trichomes on pods	Seed coat colour	Seed size	100 seed weight (g)	Seed yield/ plants (g)	L-Dopa in seed (%)
1	ZFD 101	-	White	Bold	103.34	174.98	3.16
2	ZFD 102	-	Black	Bold	161.96	270.86**	3.12
3	ZFD 103	-	White	Medium	84.03	175.82	3.48
4	ZFD 104	-	Black	Bold	174.62	187.94	2.30
5	ZFJ 106	-	Striped	Bold	206.03	264.50**	4.09
6	ZFP 110	-	White	Bold	108.08	190.16	3.16
7	ZFV 111	-	White	Bold	107.27	236.08	3.88
8	ZFA 112	-	White	Bold	108.26	208.24	4.01
9	ZFL 113	-	White	Bold	112.82	233.14	3.73
10	ZFL 114	-	Striped	Bold	119.06	245.86	4018
11	AW 100	+	Striped	Small	30.01	72.68	4.96
12	NW 101	+	Striped	Small	37.00	64.18	5.36*
13	SW 102	+	Striped	Small	24.16	91.22	5.65*
14	KSW 103	+	Striped	Small	44.12	93.26	6.12*
15	MW 104	+	Striped	Small	29.00	93.66	4.36
16	KHW 105	+	Striped	Small	28.97	110.54	5.63*
17	KGW 109	+	Grey	Small	28.56	73.32	5.36*

(Source: Krishnamurthy et al., [27])

Sixty nine (69) F_1 hybrids were developed and evaluated for seed yield and L- Dopa content. Twenty four (24) promising lines have been initially shortlisted and presented (Table 6).

The non- itching trichomes lines namely ZFD 102, ZFJ 106, ZFV 111, ZFA 112, ZFL 113 and ZFL 114 have exhibited improved seed associated characters and higher value of seed physicochemical parameters with 80% seed germination and over 3.0 % L-Dopa content. Seeds are white, black or striped in colour, bold in size, weighing 107-161 gm/100 seeds and gave 208-270 gm seed yield/plant with improved related agronomical trails. These lines were short- listed for commercial production and crop improvement.

Of these short listed lines, 23 lines have been devoid of itching trichomes on pods with White/Striped colour seed size weighing 86-250 gm/100 seeds. All the shortlisted lines were found to have above 200gm seed yield/plant with more than 4% L-Dopa in seeds. Out of the 24 lines, 7 lines have showed improved seed yield of 300-532 gm/plant and 4.38-5.66% L-Dopa and devoid of itching trichomes on the pods. Maximum L- Dopa content of 6.0-7.65% was recorded in 3lines having produced moderated seed yield (222-298gm/plant). Three lines with desired characters have been shortlisted from 14 BC lines (Table 8), and their seed and pod associated characters are presented in Table 8. All the three lines selected possessed white coloured seeds and devoid of itching trichomes on pods [27].

Chemical composition of seeds of three selected back-cross lines was estimated to find out the best suitable line for further use. Total ash content was found to be 2.5-3.0% among the three lines studied. All three lines were rich in carbohydrates (56.0-58.5), protein (25-27%), fat (5-6%) and crude fibres (0.02-0.59%). 352-389 (K.cal/100 gm) caloric value range was noticed in all three lines. These three lines have exhibited wide range of calcium (204-253 mg/100gm), iron (117-453 mg/kg) and Na (167-261 mg/kg) in the seeds. The cross of ZFV 111× KGW 109 has showed high level of

calcium (321 mg/100 gm), iron (453 mg/kg) and crude fibres (0.59%) when compared to other two lines.

Table 6. Seed associated characteristic of 24 hybrids (F_1) of *M. pruriens* shortlisted from 69 F_1 lines developed during the breeding programme

Sr. No.	F_1 crosses	Trichomes on pods	Seed colour	Seed size	100 seed wt (gm)	Seed yield/ plant (gm)	L-Dopa in seed (%)
1	ZFD 101 X ZFA 112	-	White	Bold	123.51	203.98	4.20
2	ZFD 103 X ZFD 101	-	White	Medium	104.01	298.60	5.12
3	ZFD 103 X ZFD 102	-	White	Bold	103.22	237.68	4.00
4	ZFV 111 X ZFD 101	-	White	Bold	119.65	235.44	4.69
5	ZFP 110 X ZFA 112	-	White	Bold	102.03	235.64	4.50
6	ZFV 111 X ZFA 112	-	White	Bold	116.90	227.44	4.05
7	AW 100 X KHW 110	-+	Striped	Small	35.50	286.68	5.57
8	ZFD 101 X NW 105	-	White	Bold	113.25	214.16	4.38
9	ZFD 103 X MW 101	-	White	Bold	137.50	207.08	5.04
10	ZFD 103 X MW 104	-	White	Bold	106.00	246.32	6.00
11	ZFJ 106 X NW 101	-	Striped	Bold	193.00	395.75	4.41
12	ZFJ 106 X KHW 105	-	Striped	Bold	176.75	222.60	7.65
13	ZFJ 106 X KGW 109	-	Striped	Bold	232.00	286.20	5.30
14	ZFP 110 X KSW 103	-	White	Bold	250.00	253.60	5.36
15	ZFV 111 X NW 101	-	White	Bold	133.00	349.08	4.83
16	ZFV 111 X SW 102	-	White	Medium	97.00	226.44	4.26
17	ZFV 111 X MW 104	-	White	Bold	127.50	295.02	5.67
18	ZFV 111 X KGW 109	-	White	Bold	103.50	532.24*	5.66
19	ZFA 112 X NW 101	-	White	Medium	86.00	491.62*	5.57
20	ZFA 112 X KSW 103	-	White	Medium	88.60	302.62	4.45
21	ZFA 112 X MW 104	-	White	Medium	91.00	340.10	4.38
22	ZFA 112 X KGW 109	-	White	Bold	115.60	298.74	6.02
23	ZFL 113 X MW 104	-	White	Bold	119.50	341.52	5.35
24	ZFL 114 X NW 101	-	Striped	Bold	133.00	208.44	4.35

-&+: Indicates no-itching and reduced intensity of itching trichomes on pods
(Source: Krishnamurthy et al., [27])

The cross ZFV 111× KGW 109 showed superior characteristics over the other two and it was further tested to standardize agronomic practices i.e. effect of different combination of fertilizers and effect of spacing on growth and yield (Table 9).

Twelve different combinations of NPK fertilizers have been studied and found that yield was raised through increase in number of pods/plot (9 Sq.M.) over the control. However, there was no significant difference in size and weight of seed/pod. The seed yield increased from 1.196 to 1.531 M. tonnes/ha. When the fertilizers were given at 75:100:0 (1.196 M. tonnes seed yield/ha) and 150: 50: 0 kg (1.476 M. tonnes seed yield /ha) NPK/ha depending upon N-status of the soil (Table 10).

Three types of supporting systems(viz. individual plant support, line support and wire- net support)have been studied along with control (Table 11), The study revealed that the maximum seed yield of 1.2-1.3 kg/plot with individual support, which is estimated to produce 1.420 M. tonnes/ha seed yield over other supporting system (0.695-0.710 M. tonnes/ha) and control (0.501 M. tonnes/ha.) it is evident that providing support to individual plant with bamboo stales is ideal in commercial cultivation.

Table 7. Seed associated characteristics of 14 important desirable lines of *M. pruriens* obtained through selection from 69 backcross lines

Selection Type	Line code	Cross	No. of Twinner/ Plant	No. of inflorescence/ Plant	No. of pod/plant	Trichomes on pods	Seed colour	100 seed wt. (gm)	Seed yield/plant (gm)
1. Disease Resistance to Rust & Blight	0603	ZFV-111 X KGW-109	2	34	154	-+	White	72	424
	0619	ZFV-111 X KGW-109	1	38	175	-	Green	62	354
	0512	ZFV-111 X MW-104	2	55	112	-	White	80	328
2. High seed yielding type	1305	ZFA-112 X ZFD-101	3	55	166	-+	White	102	810*
	1604	ZFV-112 X AW-100	3	51	120	-+	White	114	674*
	0203	ZFD-101 X KGW-109	2	54	173	-+	White	104	572*
	0204	ZFD-101 X KGW-109	3	61	138	-+	White	116	518*
3. Early pod maturity type	0103	ZFD-101 X MW-104	2	52	125	-	White	106	470
	0323	ZFD-103 X KHW-105	2	29	102	-+	White	110	450
	0804	ZFD-103 X ZFD-102	2	35	81	-+	White	102	380
	1304	ZFA-112 X ZFD-101	1	25	98	-+	Black	110	488
	1414	ZFD-112 X ZFD-102	1	39	82	-	White	100	354
4. Synchronous pod bearing type	1405	ZFD-112 X ZFD-102	3	26	102	-+	White	104	478
	0903	ZFD-114 X ZFD-102	2	22	82	-+	Striped	98	314

(Source: Krishnamurthy et al., [27])

Table 8. Seed associated characters of 3 important desirable backcross (BC) lines of *M. pruriens* shortlisted and selected

Sr. no.	Character	Back cross lines & line code		
		ZFV 111 XKGW 109 (0603)	ZFA 112 X ZFD 101 (1305)	ZFV 112 X AW 100 (1604)
	Selection	**Disease resistance**	**High seed yielding**	
1	Trichomes on pods	-	-	-+
2	Seed colour	White	White	White
3	Seed size	Medium	Bold	Bold
4	100 seed wt. (gm)	72.00	102.00	114.00
5	No. of pods/plants	154.00	166.00	120.00
6	Seed yield/plant (gm)	424.00	810.00	647.00

-&+: Indicates no-itching and reduced intensity of itching trichomes on pods; All the three selections are having > 4.5 % L-Dopa content (Source: Krishnamurthy et al., [27])

Table 9. Comparative account of chemical composition of three selected desirable back cross lines of *M. pruriens* developed through conventional breeding

Sr. No	Chemical composition	Line code and crosses		ZFV 111 ×KGW 109 (0603)
		ZFA 112 ×ZFD 101 (1305)	ZFV 112 × AW 100 (1604)	
1.	Moisture (%) w/w	8.65	8.52	7.84
2.	Total Ash (%) w/w	2.57	2.74	3.09
3.	Protein (%) w/w	25.98	26.09	27.38
4.	Fat (%) w/w	5.27	5.23	6.24
5.	Crude fibre (%) w/w	0.02	0.049	0.59
6.	Carbohydrates (%) w/w	58.43	58.59	56.36
7.	Calorie value (K. Cal./100 gm)	385.07	352.95	398.92
8.	Ca (mg/100 gm)	353.05	204.44	321.59
9.	P (mg/ 100 gm)	254.27	255.17	256.96
10.	Fe (mg/ kg)	117.20	123.53	453.40
11.	K (mg/ kg)	10.08	10.27	12.44
12.	Na (mg/ kg)	261.70	185.93	167.12

(Source: Krishnamurthy et al., [27])

Table 10. Effect of NPK fertilizers on yield associated characteristics of *M. pruriens*

Treatment N: P: K kg/hectare	No of pods/9 sq. mtr		Wt of seed pod (gm)		Seed yield /9sq.m. (gm)		*Calculated Yield/ha (kg)
Crop year	2000-01	2001-02	2000-01	2001-02	2000-01	2001-02	
T1 0:0:0	133.33	121.00	4.57	4.44	610.66	537.66	637.95
T2 0:50:0	179.00	156.66	4.58	4.50	828.33	705.33	852.03
T3 0:100:0	192.00	178.66	4.40	4.55	845.66	814.66	922.40
T4 75:0:75	198.00	190.66	4.97	4.71	984.00	898.66	1045.92
T5 75:50:0	236.33	224.33	4.41	4.31	1040.00	968.66	115.92
T6 75:100:0	252.00	240.00	4.36	4.36	1106.66	1047.66	1196.84
T7 150:0:0	191.00	179.66	4.53	4.39	859.00	791.66	917.03
T8 150:50:0	280.00	244.66	4.97	5.17	1390.66	1267.00	1476.47
T9 150:100:0	287.33	260.00	4.85	5.01	1391.00	1303.00	1496.85
T10 225:0:0	181.33	176.66	4.32	4.23	789.00	747.33	853.51
T11 225:50:0	277.33	251.6	4.98	5.24	1387.33	1320.00	1504.07
T12 225:100:0	295.33	268.33	4.82	4.94	1430.66	1325.66	1531.28
CD at 5%	62.9	21.66	0.535	0.396	320.67	111.459	

(Source: Krishnamurthy et al., [28])

Table 11. Effect of various support or stakes on yield-associated characteristics of velvet bean (*M. pruriens*)

Treatment	No. of pods / 9 sq.m		Seed yield/pod (g)		Seed yield/ 9 sq.m (g)		Estimated seed yield/ha (kg)
	2000-2001	2001-2002	2000-2001	2001-2002	2000-2001	2001-2002	
T1, Without support: (control)	128.38	153.04	3.90	3.80	420.00	482.70	501.50
T2, Individual plant support through bamboo poles	435.25	360.00	4.90	4.80	1242.36	1314.90	1420.70
T3, Line support	297.79	305.15	3.80	4.00	562.90	715.50	710.22
T4, Wire net support	300.54	203.03	4.98	4.70	653.23	598.5	695.40
CD (P=0.05)	128.39	40.05	0.52	0.3646	210.36	222.97	

(Source: Krishnamurthy et al., [28])

Table 12. Effect of various stages of pod harvesting on seed yield associated characteristics on velvet bean (*M. pruriens*)

Treatment	Fresh weight of pod (g)	Dry weight of pod (g)	Total moisture content of pods (%)	Days to complete drying of pods	Time taken in removing seeds from pods (sec)	Dry seeds weight (g)	L-Dopa in seeds (%)	Seed quality
Stage of developing pods T1 (green)	736.40	189.40	74.27	14.00	167.50	107.80	4.722	> 50 seeds blackish wrinkled and with cracks
T2 (yellowing)	431.40	194.60	54.89	8.00	128.00	113.60	5.004	> 50 seeds blackish White and superior quality
T3 (semi-arid)	257.40	180.80	25.82	7.00	164.00	110.40	4.680	White and superior quality
T4 (dried)	224.60	188.60	15.79	5.00	165.00	110.40	3.961	White and superior quality
CD (P=0.05)	31.30	15.16	5.10	9.30	8.95			

(Source: Krishnamurthy et al., [28])

Table 13. Effect of biofertilizers on yield and quality of *M. pruriens*

Treatment	No of pods/ plot (9sq.m)		Seed yield (kg/ha.)	L-Dopa (%)
Years	**2001-01**	**2001-02**	**Average of 2 years**	
T_1	128.00	143.66	626.85	3.66
T_2	254.00	243.66	1169.25	3.69
T_3 (Bio- mix)	144.33	169.33	781.84*	3.41
T_4	290.00	296.00	1576.67	3.99
T_5 (Bio-mix+ low NPK)	266.53	248.68	1260.55*	3.67
T_6	180.00	198.33	1001.43	3.43
T_7	270.00	288.00**	1424.81	3.65
T_8(Bo	206.00	219.66	1132.77*	3.57
T_9	168.00	186.66	909.62	3.33
CD at 5%	73.00	49.62	-	-

(Source: Krishnamurthy et al., [29])

Four stages of developing pods viz. green (early mature), turning yellow (mid mature), semi- drying and fully drying have been assessed for seed associated characters and seed quality (Table 12, Fig. 5 and 6). An analysis of result showed that seeds harvested from green (T1) and yellowing (T2) pods have developed 50% blackening, wrinkled and showed cracks during drying. On the contrary, better quality seeds (in appearance, shape and colour) were recorded from pods harvested at semi drying (T3) and late-drying stages (T4); theses weighed about 110 gm/100 seeds. Similarly, the days taken for drying and efficiency of separating seeds from pods have not significantly varied between T3 and T4 treatments (Table 10). On the whole, optimum L-Dopa content of 4.684% with better appearance of seeds was recorded in T3 stage (semi-dried stage); The L-Dopa content was found reduced (3.961%) in T4 stage of drying pods when compared to other stages. This study indicate that the right stage of harvesting of pods is at semi drying stage (T3) for optimum yield, higher L-Dopa content and superior quality of seeds for drug manufacturing industry in commercial cultivation [28].

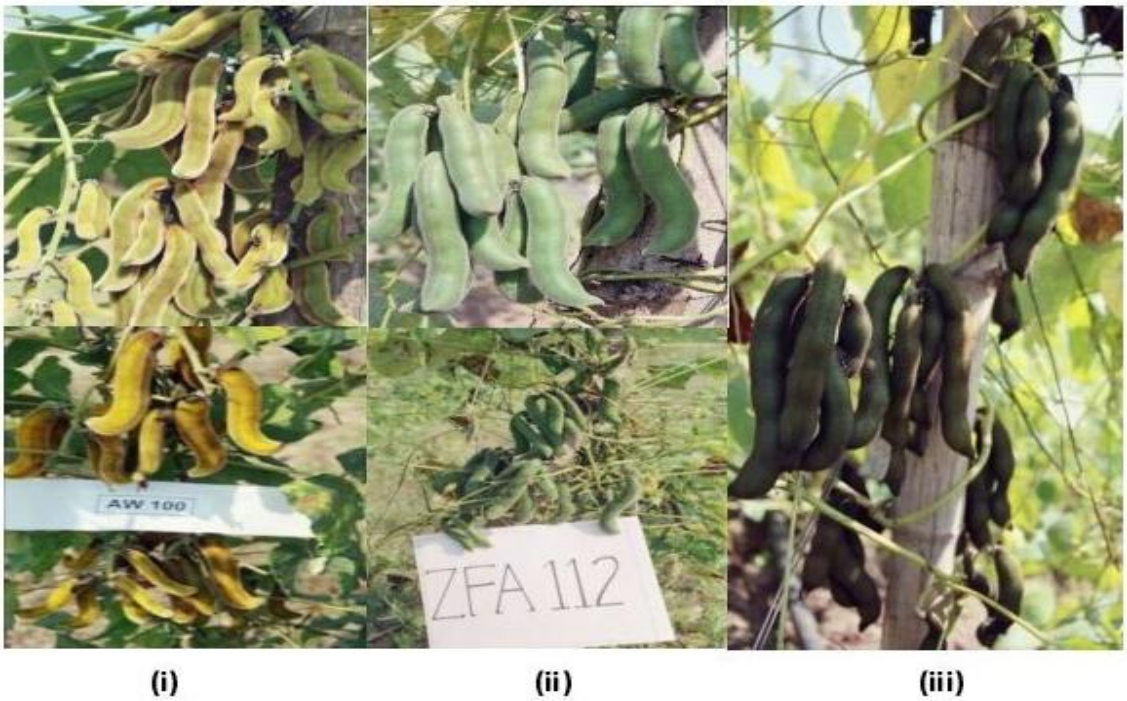

Fig. 5. *M. pruriens* high yielding seed lines, (i) Brown pods with striped bold seed lines with less itching trichomes on pods; (ii) Green pods of white coloured bold seed lines with non-itching trichomes; (iii) Black coloured bold seed line with non-itching trichome on pod

Fig. 6. *M. pruriens* breed line with synchronous drying of pods and ready for harvesting

Mucuna pruriens (Velvet bean) :
Farmers are engaged in sun drying of pods.

Fig. 7. Separating seeds from *M. pruriens* capsule for commercial seed production

The cost benefit ratio (C:B) in Mucuna showed higher values (1.055) for treatment where chemical fertilizers alone is used and showed significant increase (1.462). It is much lower when the biofertilizer alone was applied (0.555) or in control where FYM alone was applied (0.299) (Table 13). The study on bio-fertilizers lead us to believe that its use has significant contribution in improving yield, reducing cost of production and step up fertility status of the land in long-run (Fig. 8) [29].

Fig. 8. *M.* pruriens cultivation field after harvesting of pods. The dry leaves and whole dry plants were mulched in the soil as manure

4. CONCLUSION

M. pruriens is the important medicinal and forage legume cultivated in the tropical countries. Mucuna seeds are well known for its enormous therapeutic properties and it has high demand in pharmaceutical industries, although its cultivation is restricted due to the itching trichomes present on pods. Collection of wild plant materials also revealed that the most of the accessions have low yield associated, seed yield characters as well as very low - moderate L-Dopa content in seeds. Individually, wild accessions are less attractive for the commercial cultivation and therefore, large scale cultivation of *M. pruriens* lines necessitates the development of improved cultivars. This is the first report on development of non-itching lines with higher seed yield and L-Dopa content from the wide range of itching wild accessions [27]. Further, the standardization of agronomic practices [28,29] revealed that these breed lines have all superior characteristics i.e. medium sized seeds with 1.420 M. tonnes/ha seed yield and above 4.5% L-Dopa and it is suitable for the commercial cultivation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 4
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Accelerated Stability Study in Alternative Formulations of a Second- Generation Biopharmaceutical Product

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ABSTRACT

Filgrastim, a granulocyte colony stimulating factor (G-CSF), is a hematopoietic glycoprotein that binds with high affinity to its receptors in neutrophil precursor cells of the bone marrow; thus inducing proliferation and differentiation in neutrophils. The first-generation commercial product has a short half-life (between 3.5 to 3.8 hours). However, this half-life can be extended by means of a covalent modification with PEG (polyethylene glycol). Pegylation increases the hydrodynamic volume of the molecule, minimizing renal clearance. Thus, the use of pegylated molecule is more advantageous than the administration of several doses of "naked" protein. Each pre-filled syringe of the original formulation contains 6 mg of pegfilgrastim in 0.6 ml of solution for injection, one of its components is sorbitol, a widely used tonicity modifier. In this work an alternative formulation is proposed, replacing sorbitol with mannitol, which is mostly used in lyophilized formulations because it crystallizes easily. In this study we propose to start the comparative evaluation of two formulations (FO: original and FA: alternative) of pegylated Filgrastim (PF) through a brief study of accelerated stability consisting in the exposition of both formulations to different temperature and freezing / thawing conditions. Subsequently both its structure and its stability were analyzed through different spectroscopies. For the evaluation of the structural conformation in the times established (zero time, one week and one month) both formulations were analyzed by UV spectroscopy, circular dichroism and fluorescence. Possible stability alterations were monitored by a thermal denaturation test. Both formulations of PF were aliquoted and stored at 4°C, 25°C and 37°C. Another group of samples were kept at extreme temperatures (-80°C, -20°C, -4°C and 57°C). At the time of carrying out the different measurements, the necessary dilutions were made in FO/FA. Regardless of time and storage temperature, samples formulated with mannitol or sorbitol did not show changes in secondary structure content or alterations in tertiary structure. Also, we did not find alterations in its conformational stability. These preliminary studies highlight the viability of the replacement of mannitol by sorbitol in the PF formulation, since the analyzes carried out through the proposed methods did not demonstrate protein instability linked to the change in formulation.

Keywords: Stability; formulation; protein; circular dichroism.

ABBREVIATIONS

ABS : Absorption
CD : Circular Dichroism
CEx-HPLC : Cation Exchange - High Performance Liquid Chromatography

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CPMP : Committee for Proprietary Medicinal Products
ESI-MS : Electrospray Ionization
FA : Alternative Formulation (with mannitol)
FO : Original Formulation (with sorbitol).
G-CSF : Granulocyte Colony Stimulating Factor
HDX : System w/Hydrogen Deuterium Exchange
HR : Relative humidity
PEG : Polietilenglicol
PF : Pegfilgrastim
pH : Hydrogen Potential
RAN : Absolute Neutrophil Count
RP-HPLC : Reverse Phase High Performance Liquid Chromatography
RP-UPLC : Reverse Phase Ultra Performance Liquid Chromatography
SDS-PAGE : Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SE-HPLC : Size-Exclusion-High Performance Liquid Chromatography
UV : Ultraviolet

1. INTRODUCTION

Granulocyte colony stimulating factor (G-CSF) is a hematopoietic glycoprotein produced by stromal cells, macrophages, endothelial cells, fibroblasts and monocytes. This protein binds with high affinity to the G-CSF receptor expressed in bone marrow neutrophil precursor cells thus inducing proliferation and differentiation in neutrophils fighting infection. This factor has no significant hematopoietic effects in other blood cell lineages.

The formulation with G-CSF is indicated as a treatment to accelerate the recovery of the bone marrow, thus preventing the onset of severe myelosuppression. It is also used to achieve a reduction in febrile neutropenia in patients with non-myeloid or chemotherapeutic malignancies. The commercial product Filgrastim (Neupogen) has a relatively short half-life (between 3.5 to 3.8 h, but levels may vary based on neutrophil count) so they need a daily dose. Despite this, the half-life of the recombinant protein can be increased by means of a covalent modification with PEG (polyethylene glycol), an inert and hydrophilic polymer by a process called "Pegylation" [1].

Pegylation is a widely used procedure to improve the pharmacological profile of various agents, improving the biophysical properties of the protein and increasing the solubility. PEG molecules are non-toxic because they do not interact with biological structures, tissues or organs; they are flexible and generate a cover around the protein, which avoids proteolytic degradation and opsonization (uptake by macrophages). Likewise, renal clearance of the PEG- associated protein is greatly reduced. The G-CSFrh-bound PEG molecule (the human recombinant form of G-CSF) does not interfere with ligand binding to the receptor, as long as the pegylation site is adequate to preserve the activity of the main molecule. In fact, numerous in vitro studies revealed that the biological activity and mechanism of action of PF (pegfilgrastim) are the same as those of filgrastim. The work in animals showed that the kidneys participate very little in the elimination of PF, which is why the purification of the drug depends almost exclusively on the RAN (absolute neutrophil count) [2].

The pharmacokinetic profile of the pegylated molecule is directly related to the size of the PEG, as well as the number of PEG chains attached to the protein. Therefore, for pegylation to be effective, it is essential to consider the PEG binding site and the chemistry used in that covalent bond. In addition, depending on the conjugation site, the molecules may show a decrease in immunogenicity compared to parenteral molecules. A successful pegylation strategy results from the balance between: (i) clinical improvement, (ii) changes in biophysical properties provided by pegylation and (iii) the potential loss of bioactivity due to steric interference with the union of the target [1].

1.1 Biopharmaceuticals and their Classification

Biological drugs or biopharmaceuticals are homologous therapeutic substances that consist of an extremely complex molecular structure and in addition to that, their active component is heterogeneous, which makes them difficult to characterize and replicate. They are classified in:

First generation: Those simple replacement proteins; that is, they have an identical amino acid sequence to the natural human protein and are administered to the body to replace or increase the levels of said protein.

Second generation: They are those that have been modified in terms of stability, efficacy, immunogenicity, specificity to optimize therapeutic properties.

Third generation: These are those that have fragments of human proteins, antibodies or peptides derived from antibodies.

The so-called colony stimulating factors (CSF) are a set of cytokines that induce the proliferation and differentiation of progenitor cells certain subpopulations of leukocytes, specifically granulocytes and macrophages. Four main types are grouped under this denomination:

- G-CSF: Granulocyte stimulating factor.
- M-CSF: macrophage stimulating factor.
- GM-CSF: Granulocyte-macrophage stimulating factor.
- Multi-CSF: Multifunctional factor (also called interleukin 3: IL-3).

CSFs not only influence the differentiation and maturation of white cell precursor cells but also intervene in the functional activity of mature cells. They have many common features in their molecular structure and in the basic mechanism of action. Perhaps the most important difference is that the influence of CSFs on genetic material is primarily translated into DNA replication, while the action of immunomodulators is translated into genetic expression (protein synthesis), in this case DNA replication a secondary phenomenon [3].

All these substances should be considered as members of a complex network of molecular messengers that regulate the immune system. Since one of the actions of cytokines is to stimulate the production of other cytokines, the final effect on the organism can be quite different from what could be predicted from its in vitro behavior.

The natural production of CSF does not appear to be directly related to the leukocyte count. Under normal conditions, blood levels of CSF are very low, regardless of the numbers of neutrophils or macrophages, but they increase considerably when there is a bacterial infection. Of the four main CSFs only two are marketed:

- granulocyte stimulating factor (G-CSF): filgrastim and lenograstim
- granulocyte-macrophage stimulating factor (GM-CSF): molgramostim

Natural CSFs are glycoproteins. The glycosylation of the molecule is easily achieved in products derived from eukaryotic cells (lenograstim), but is usually omitted for technological reasons in those derived from bacterial cultures (filgrastim and molgramostim). However, the physiological action depends exclusively on the protein chain. The differences between the glycoprotein and the pure protein fraction are of the pharmacokinetic type [3].

The difference in hematological response between G-CSF and GM-CSF does not seem to have great clinical relevance at the moment. As expected, action promotes mostly neutrophil growth, though only at high doses macrophage increase is seen. Eosinophil counts are not affected. GM-CSF produces a significant increase in neutrophils, but they also increase macrophages and eosinophils. The rest of the white cells are not modified in any case [3].

It is advisable to use G-CSF in situations characterized by neutropenia, while the usefulness of GM-CSF (and other more versatile factors, such as multi-CSF) would be in cases of bone marrow dysfunction causing a leukocyte type deficiency. In practice, the two types of CSF marketed are being applied in the same indications [3].

Despite all the variety of colony stimulating factors mentioned above, the product of analysis on which our work is based is the PF. This corresponds to a second-generation drug, since a PEG was added to the original molecule. As mentioned earlier, this process can improve not only the administration (reducing the number of administrations required), but also modulate the distribution of the cytokine inside the organism, thus improving efficiency and, eventually, reducing its toxicity. Pegylation usually affects the physicochemical properties of cytokine, generally modifying its solubility. However, it does not affect its primary, secondary or tertiary structure [4].

Based on the aforementioned, Neulasta (Pegfilgrastim) was produced by binding a 20 kDa PEG to the α-amino group of the N-terminal methionine residue of Filgrastim [5]. Modification with PEG increases the hydrodynamic volume of the molecule causing this product to have a longer renal clearance. The mechanism of clearence in humans can occur in two ways: renal clearence and neutrophil-mediated clearence. After administration of the PF to patients, their serum concentration remains elevated during neutropenia, but decreases when the neutrophil count increases. Thus, the use of pegylated molecule is more advantageous than the administration of several doses of filgrastim.

Each pre-filled syringe of the original formulation contains 6 mg of PF in 0.6 ml solution for injection, one of its components being sorbitol, which is a widely used tonicity modifier. However, in the present work, an alternative formulation is proposed, making use of mannitol [6], which is mostly used in lyophilized formulation because it easily crystallizes [7]. Sorbitol was used in an alternative formulation to verify the stability of the protein against this change in formulation.

2. OBJECTIVE

Comparative evaluation of the two formulas (original and alternative) of PF through an accelerated stability study focused on subjecting both formulations to different conditions of temperature, freez-thawing and subsequently analyzing both its structure and its conformational stability through different spectroscopies.

3. THEORETICAL FRAMEWORK

3.1 Stability

Stability tests are necessary throughout the life cycle of a product; because the results help analysts understand how critical quality attributes of drug substances and products are influenced by specific conditions of temperature, relative humidity (RH), light, storage, pH and other factors. Stability tests should be performed to determine degradation pathways, establish shelf life and storage conditions of products. Tests and bioassays related to purity, identity, potency, quality and safety are carried out according to the type of product and the intended use [8].

3.2 Regulatory Requirements

Regulatory agencies typically require stability tests for products. For this, there are certain guidelines such as the ICH (International Conference on Harmonization), where the ICH Q5C, specifically refers to biological products. There are also general chapters of USP (United States Pharmacopeia) <1049> and <1046> that provide general considerations for biopharmaceuticals and cellular and genetic therapies.

EMA (European Medicines Agency) guidelines include CPMP (Committee for Proprietary Medicinal Products) / QWP (Quality Working Party) / 609/96:

- Declaration of storage conditions (an annex to the stability guidelines that apply to the declarations of storage conditions in the labeling);
- CPMP / QWP / 2934/99: Stability tests in use (applies to products in multi-dose containers);

- CPMP / QWP / 159/96: Maximum shelf life for sterile products after first opening or after reconstitution (which says that users are responsible for maintaining the quality of a product that is administered to patients and provides guidance on the information text for the user).

Other organizations, such as the WHO (World Health Organization) and the Association of Southeast Asian Nations, have published stability testing guidelines for both finished products and active pharmaceutical ingredients (APIs), but some apply only to conventional medications (small molecules) [8].

3.3 Technologies and Protocols

Although stability tests are covered in several guidelines, those documents do not provide details on how to perform the tests they recommend. Analysts must develop methods and bioassays and then validate them as methods that indicate stability. Thus, a stability check protocol is developed according to what needs to be determined. They also have to indicate when specific methods should be carried out; having to ensure that those detect possible changes in the identity, purity and potency of a product [8].

Table 1. ICH Q1A guidelines for storage. Medications should be evaluated under storage conditions

Case	Study	Conditions	Minimum Time[1]
General	Long term[2]	25±2°C/60±5% RH; 30±2°C/65±5% RH	12 months
	Intermediate[3]	30±2°C/65±5% RH	6 months
	Acelerated	40±2°C/75±5% RH	6 months
Stored in refrigeration	Long term	5±3°C	12 months
	Intermediate	25±2°C/60±5% RH	6 months
Stored in freezer	Long term	-20±5°C	12 months

Note.[1] Minimum period of time covered by the data at the time of submission
[2]It is up to an applicant to decide whether long-term stability studies are conducted at 25 ± 2°C and 60 ± 5% RH or 30 ± 2°C and 65 ± 5% RH
[3]If the long-term condition is 30 ± 2°C and 65 ± 5% RH, then there is no intermediate condition

As noted above, the expiration date should be based on data obtained in real time/real temperature. However, it is also advisable to conduct studies on the pharmaceutical substance and the pharmaceutical product under conditions of stress and acceleration [8].

Accelerated studies can provide useful supporting data to establish the expiration date, provide information on the stability of the product for future development (for example, a preliminary assessment of the proposed manufacturing changes, such as the change in formulation, the extension), assist in the validation of analytical methods for the stability program, or generate information that can help elucidate the degradation profile of the drug substance or product. On the other hand, studies in stressful conditions can be useful to determine if accidental exposures to conditions other than those proposed (for example, during transport) are harmful to the product and also to evaluate what specific test parameters may be better indicators of product stability [8].

Studies on the exposure of the substance or drug product to extreme conditions can help reveal patterns of degradation; if so, such changes should be monitored under the proposed storage conditions. While the tripartite guide to stability describes the conditions of the accelerated and stress study, the applicant should keep in mind that those conditions may not be suitable for biotechnological / biological products. The conditions must be carefully selected on a case-by-case basis [8].

3.4 Filgrastim

3.4.1 Discovery

The substance was discovered at the Walter and Eliza Hall Institute in Australia in 1983, isolating itself from laboratory mice. Subsequently, the human molecule was discovered that was cloned by different groups of researchers in Japan, Germany and the United States in 1986 [9].

3.4.2 Structure

Filgrastim is a human granulocyte colony stimulating factor produced by genetic recombination technology. G-CSF is produced industrially by an *Escherichia coli* in which the human gene encoding G-CSF has been inserted. The protein obtained, of a molecular weight of 18800 Daltons, is identical to the human protein except for of the addition of a single methionine in the amino terminal, necessary for the protein to be expressed by *E. coli*. In addition, unlike the natural protein, recombinant filgrastim is not glycosylated [10].

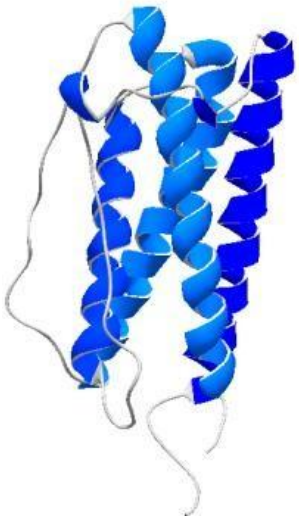


Fig. 1. Structure of filgrastim (PDB 1CD9)

MET THR PRO LEU GLY PRO ALA SER SER LEU PRO GLN SER
PHE LEU LEU LYS CYS LEU GLU GLN VAL ARG LYS ILE GLN
GLY ASP GLY ALA ALA LEU GLN GLU LYS LEU CYS ALA THR
TYR LYS LEU CYS HIS PRO GLU GLU LEU VAL LEU LEU GLY
HIS SER LEU GLY ILE PRO TRP ALA PRO LEU SER SER CYS
PRO SER GLN ALA LEU GLN LEU ALA GLY CYS LEU SER GLN
LEU HIS SER GLY LEU PHE LEU TYR GLN GLY LEU LEU GLN
ALA LEU GLU GLY ILE SER PRO GLU LEU GLY PRO THR LEU
ASP THR LEU GLN LEU ASP VAL ALA ASP PHE ALA THR THR
ILE TRP GLN GLN MET GLU GLU LEU GLY MET ALA PRO ALA
LEU GLN PRO THR GLN GLY ALA MET PRO ALA PHE ALA SER
ALA PHE GLN ARG ARG ALA GLY GLY VAL LEU VAL ALA SER
HIS LEU GLN SER PHE LEU GLU VAL SER TYR ARG VAL LEU

ARG HIS LEU ALA GLN PRO

Fig. 2. Filgrastim amino acid sequence (PDB 1CD9)

3.4.3 Biological functions

As Furmento [11] indicates, G-CSF is produced by different body cells. This molecule exists in two forms that consist of 175 and 180 amino acids. The most abundant and active form is that of 175

amino acids, which has been used in the development of pharmaceutical products using DNA recombination technology.

The G-CSF receptor is present in the bone marrow precursor cells and, when stimulated, produces in response the proliferation and differentiation of the precursor cells that become mature granulocytes. G-CSF also induces the mobilization of granulocytes that, starting from the bone marrow, are directed to the bloodstream. In addition to the effects on the hematopoietic system, G-CSF can also act in neurons as a neurotrophic factor. In fact, its receptor is expressed by neurons of the brain and spinal cord. The action of G-CSF in the central nervous system is to induce neurogenesis, to increase neuroplasticity and counteract apoptosis. These properties are currently being investigated for the treatment of neurological diseases such as cerebral ischemia [11].

3.4.4 Quali-quantitative formula

The pre-filled syringes together with the single-use vials contain 300 mcg or 480 mcg of filgrastim in a filling volume of 0.5 ml or 0.8 ml, in the pre-filled syringes and in a volume of 1.0 ml or 1.6 ml in vials. Below is a table that shows the composition of the different products.

Table 2. Quali-quantitative formulation of Filgrastim-Neupogen Reference ID: 3136501 [12]

	300 mcg/ 1.0 ml Vial	**480 mcg/ 1.6 ml Vial**	**300 mcg/ 0.5 ml Syringe**	**480 mcg/ 0.8 ml Syringe**
Filgrastim	300 mcg	480 mcg	300 mcg	480 mcg
Acetate	0.59 mg	0.94 mg	0.295 mg	0.472 mg
Sorbitol	50.0 mg	80.0 mg	25.0 mg	40.0 mg
Polysorbate 80	0.04 mg	0.064 mg	0.02 mg	0.032 mg
Sodium	0.035 mg	0.056 mg	0.0175 mg	0.028 mg
Water for injection USP q.s.	1.0 ml	1.6 ml	0.5 ml	0.8 ml

3.4.5 Therapeutic use

G-CSF stimulates the production of neutrophil precursor cells. In hematology and oncology the recombinant form of this glycoprotein is used to facilitate the recovery of cancer patients suffering from neutropenia as a side effect of chemotherapy; because it causes myelosuppression, causing too low levels of white blood cells in the blood, which can cause severe infections and sepsis [11].

3.4.6 Biosimilars

Sandoz's biosimilar filgrastim is marketed under the brand name 'ZarzioR' in more than 30 countries outside the US [13].

The original product, Neugen of Amgen (filgrastim), was approved by the FDA (Food and Drug Administration) in February 1991. Neupogen had worldwide sales of US $ 1.4 billion in 2013 before the approval of the first biosimilars of filgrastim.

Neupogen patents expired in the USA. In December 2013 and in Europe in 2006. Some of the filgrastim biosimilars approved by the EMA are currently the following [14,15]:

Accofil (Accord Healthcare)
Filgrastim Hexal (Hexal AG)
Grastofil (Apotex Europe BV)
Nivestim (Pfizer Europe MA EEIG)
Ratiogastrim (Ratiopharm GmbH)
Tevagastrim (Teva GmbH)
Zarzio (Sandoz GmbH)

On the other hand, the biosimilars approved by the FDA are: Nivestym (July 2018) and Zarxio (March-2015) [16].

Today in Argentina, Neutromax is marketed, by Biosidus and Filgen, by Bioprofarma.

3.5 Pegfilgrastim

3.5.1 Structure

As mentioned by Esguerra [17], the PF is formed by the covalent chemical conjugation of filgrastim with a monomethoxy-PEG propionaldehyde chain, anchored in the amino terminal of the filgrastim, leading to a molecule with a weight of 38.8 kDa.

The conjugation with a PEG chain causes filgrastim to have an excessively large molecular weight to allow it to be filtered in the renal glomeruli. This determines that the PF cannot be eliminated by the renal route, the only way of clearence is though cellular metabolism of the neutrophils themselves. Consequently, in neutropenic patients this clearance will be significantly lower than in healthy subjects, and determines that the elimination of PF is, to some extent, self-regulated, facilitating high concentrations of PF during the neutropenia phase, but reducing these concentrations as the Neutrophil population is normalized.

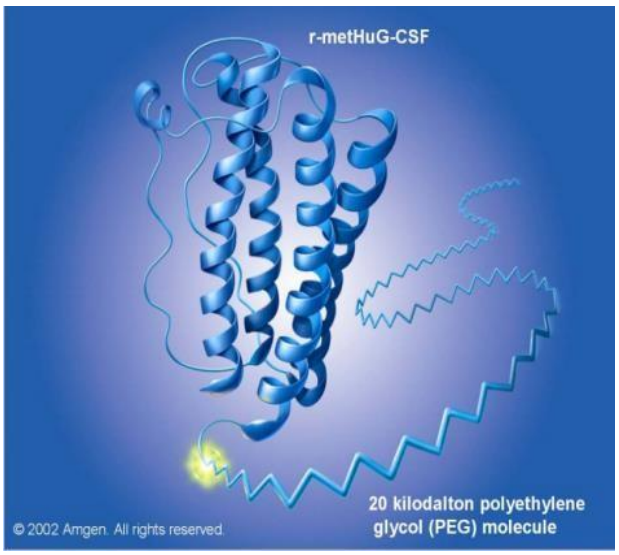


Fig. 3. Pegfilgrastim protein structure

3.5.2 Quali-quantitative formula

Acetic Acid	0.363 mg
Sodium Hydroxide	0.035 mg
Sorbitol	30 mg
Polysorbate 20	0.022 mg
Water for injectables q.s.	0.6 ml

Each pre-filled syringe contains 6 mg of pegfilgrastim in 0.6 ml of solution for injection. The concentration is 10 mg / ml referred only to protein.

3.5.3 Mechanism of action

As Esguerra [17] says, filgrastim and PF have the same mechanism of action, they bind to specific receptors for human G-CSF present in neutrophils and neutrophil precursors of bone marrow and peripheral blood stimulating their proliferation and differentiation causing a marked increase in neutrophils in peripheral blood in 24 hours, with minimal elevations of monocytes and/or lymphocytes. Like filgrastim, neutrophils produced in response to PF have normal functionality as evidenced by chemotaxis and phagocytic function tests.

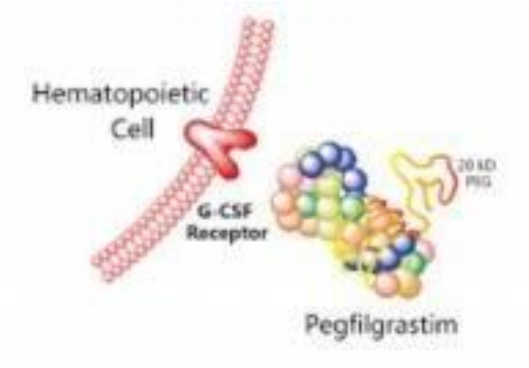


Fig. 4. Mechanism of action. Pharmacodia, pegfilgrastim (2017)

3.5.4 Biosimilars

The Amgen Neulasta product (PEG) was approved by the FDA in January 2002 and by the EMA in August 2002 [18].

Neulasta patents expired in the US in October 2015 and in Europe in August 2017 [18].

Pelgraz, a pegylated G-CSF biosimilar, is the latest addition to the established Accord Healthcare portfolio of more than 30 oncology treatments in Europe.

The Committee for Medical Products for Human Use (CHMP) has issued a positive opinion for Pelgraz (PF) of Accord, a biosimilar stimulating factor of pegylated granulocyte colonies, licensed in the EU that has phase III clinical data in addition to the phase I as part of its efficacy and safety profile, indicated to reduce the duration of neutropenia and the incidence of febrile neutropenia for adult patients undergoing cytotoxic chemotherapy [19].

The EMA has recommended the authorization of five pegfilgrastim biosimilars: Pelmeg (Cinfa Biotech), Fulphila (Mylan), Ziextenzo (Sandoz), Pelgraz (Accord Healthcare), Udenyca (ERA Consulting GmbH) [20,21,22].

The FDA has approved the biosimilars: Udenyca (November-2018) and Fulphila (June-2018).

In Argentina, the PF is marketed under neulastim- Varifarma and Peg-neutropine - GEMA biotech.

The World Health Organization considers G-CSF essential therapies due to its impact on febrile neutropenia, chemotherapy dose delays and dose density [23].

3.6 Quality Control

3.6.1 Physicochemical properties and structural characterization

3.6.1.1 Molecular mass

To determine the molecular mass of the protein in question, electrophoresis could be used in acrylamide gels in the presence of SDS (SDS-PAGE), which also allows us to show the existence of impurities [24]. Mass spectrometry analysis will also allow us to obtain information about the distribution of the molecules of a substance based on its mass.

3.6.1.2 Protein concentration

Protein content is also a key attribute since the syringes of the PF are filled based on the protein content. This is determined spectrophotometrically by correlating the absorbance at 280 nm with its calculated extinction coefficient (0.836 L. mol^{-1}. cm^{-1}) [24].

3.6.1.3 Structural analysis

As for the analysis of the primary structure, it can be determined by coupling a UV and mass spectrometry detector (eg ESI-MS) to the output of a reverse phase chromatography column (RP-HPLC). The circumvention profile should show the pattern of peaks corresponding to the main peptides obtained by enzymatic digestion of the protein under study.

The higher order structure (secondary and tertiary) is evaluated by circular dichroism spectroscopy in the UV region [24]. (See spectroscopic methods section).

3.6.1.4 Biological activity in vitro

To determine the relative potency, the measurement of biological activity in vitro is performed. This consists of a validated cell proliferation assay that utilizes the ability of an M-NFS-60 murine myeloblastic cell line to proliferate in the presence of PF in a dose-dependent manner [24].

3.6.1.5 Receiver union

The binding of G-CSF to the receptor present in human granulocytes should also be evaluated by flow cytometry. While the flow cytometry-based assay is a key measure to assess binding activity, this methodology does not provide direct information on the binding constant or on the activation and deactivation rates. For this, SPR (Resonance of surface plasmons) should be used to provide additional information on the kinetics of PF binding to isolated cellular receptors attached to the surface of a chip [24].

3.6.1.6 Purity analysis

The impurities in the sample are determined by various chromatographies such as molecular exclusion (SE), cation exchange (CEX) and reverse phase (RP), coupled to an HPLC system. In the different profiles is the main peak (composed of the active molecule) and may be accompanied by minor peaks that will correspond to different impurities. The oxidized variants are evidenced by RP, impurities related to loading in CEX and those related to protein aggregation can be observed by SE [24].

3.6.2 Optical spectroscopic methods for the study of conformational changes in proteins

The optical properties of a protein depend on the molecular environment and the mobility of its chromophores. Optical spectroscopy provides a quantity of information about the structure of proteins that is difficult to obtain by other methodologies. The absorption of UV light, emission of fluorescence and circular dichroism, among others, are suitable for the investigation of changes in the secondary, tertiary or quaternary structure of proteins [25].

3.6.3 UV absorption spectroscopy

Proteins absorb light in the near UV region (250-300 nm) due to the presence of aromatic residues: Trp, Tyr, Phe, which contribute differently to the absorption of a protein. Disulfide bridges have a weak absorption band around 250 nm that can influence the resulting spectrum. In addition, the spectrum is sensitive to the nature of the solvent, because it can interact with the chromophore (electrostatic interactions, hydrogen bridge, etc.) and alter absorption.

However, some of the spectrum information in the UV region of the proteins is masked, since the absorption bands are very wide and the changes that occur around the chromophores are small. Because of that, deriving the absorption spectrum helps identify the type of residue that is undergoing such variation, while amplifying subtle changes that are barely observed in the zero-order spectrum. Generally the second and fourth derivative of the absorption spectrum are used and this last procedure results in the highest resolution because the amplitude of the bands is inversely proportional to the average width of the original bands, consequently, the wide bands are minimized with the differentiation [25].

3.6.4 Circular Dichroism

The CD technique has a high level of sensitivity, which allows monitoring conformational changes of protein molecules. Even when there is the impossibility of a detailed structural interpretation, a change in protein structure will almost certainly be evidenced as a change in the dichroic signal [25].

The phenomenon of circular dichroism is responsible for measuring the differential absorption of two circularly polarized beams of light in the opposite direction (right, left) by optically active molecules. The difference between the extinction coefficients (ΔεL and ΔεD) is linearly related to ellipticity (θ). CD signals appear in the same spectral regions where the UV or visible absorption bands of a particular compound are found and occur when the compound itself or its environment is asymmetric. From this there are two defined regions of the CD spectrum that provide complementary information regarding the conformation of a protein: The region between 180 and 250 nm (far UV) and the region between 250 and 340 nm (near UV) [25].

3.6.4.1 Far UV region

The most important chromophore in peptides and proteins is the peptide bond itself (amide group). Depending on the particular secondary structure in which the peptide bond is immersed, these transitions will contribute differently to the CD spectrum of the macromolecule. The spectrum of a protein in this region is usually interpreted as the linear combination of spectra of typical secondary structure of type α-helix (α), folded sheet β (β) and random ball (r) in the proportion in which each of these types of secondary structure appears in the protein. Disordered structures, such as oligopeptides and denatured proteins, generally show a CD spectrum with a strong negative band near 200 nm and some weak bands between 220 and 230 nm that can be negative or positive [25].

3.6.4.2 Near UV region

Most of the proteins also have optical activity in the near UV region, mainly due to the presence of aromatic amino acids in asymmetric environments or to the torsional chirality of the disulfide bridges. This spectrum is unique (fingerprint) for each protein and cannot be interpreted simply as a linear combination of model spectra but is very sensitive to changes in tertiary structure. In addition, the fine structure and the differences in wavelengths make it possible, in certain cases, to distinguish bands corresponding to each class of aromatic amino acid residue [25].

3.6.5 Fluorescence

Fluorescence is the emission of radiant energy that occurs when a molecule in an excited state returns to its basal state of energy. The emission of light can reveal molecular properties different from the properties revealed by absorption. This process takes place on a comparatively much slower time scale, allowing a greater number of interactions and disturbances to influence the emission spectrum. In addition, fluorophores decay to excited levels of S0 resulting in additional losses of vibrational energy. For this reason, the energy of the emitted light is always less than the absorbed light, so that the fluorescence emission is displaced at longer wavelengths [25].

4. MATERIALS AND METHODS

4.1 Materials

The commercialized product that was used for the experiment was donated by a biopharmaceutical company as well as both formulation buffers (with sorbitol or mannitol).

Original formula: 10 mM acetic acid, 5% w/v sorbitol, polysorbate 20 0.003% w/v, 1.4 mM sodium hydroxide.

Alternative formula: 10 mM acetic acid, 5% w/v mannitol, polysorbate 20 0.003% w/v, 1.4 mM sodium hydroxide.

Both formulations have a pH ~ 3.5 - 4.5

4.2 Sample Preparation

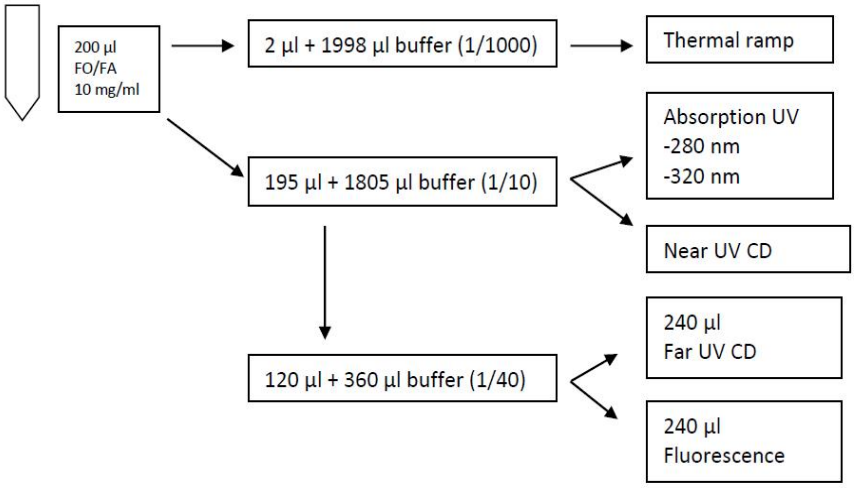
Based on the PF samples, a dialysis process (MWCO 15 KDa) was carried out followed by sterilization by filtration (0.22 µm) and storage of the samples (200 µl) in eppendorf at different temperatures: 4°C, 25°C, 37°C. Other samples were subjected to extreme temperatures (-20°C, -80°C, 57°C). Additional samples were stored for sterility controls (microbiological on LB plates) and impurity content (HPLC-RP).

4.3 Obtaining Results

To evaluate the conformation and stability of the samples, analysis of both formulations were carried out through UV absorption spectroscopy, circular dichroism (in the near and far UV region), fluorescence and thermal denaturation. These tests were performed at the beginning of the experiment, after the dialysis process and at the end of each stipulated storage time (one week and one month).

4.4 Experimental Design

Protocol for the evaluation of the structure and conformational stability of the samples.



The previous scheme summarizes the work protocol to follow each time a sample was analyzed. It consisted of taking 2 µl of the 200 µl of each sample (10 mg/ml) stored at the corresponding temperatures and diluting 1/1000 by adding 1998 µl of the corresponding buffer (FO, FA). With these samples thermal denaturation was performed, and 195 µl of the same stored sample was taken and diluted in 1805 µl of the respective buffer (1/10 dilution). The UV absorption spectrum was measured on this solution, recording the absorbance values at 280 nm and 320 nm. Next, the CD spectrum was measured in the near UV region. Finally, starting from the 1/10 dilution, 120 µl was taken and 360 µl of buffer was added. This new dilution (1/40) was divided into two: 240 µl to measure the CD in the far UV region and 240 µl to measure the intensity of the intrinsic florescence emission spectrum.

The Eppendorf consumables that were used for the storage of the samples and for the preparation of the dilutions have been manufactured without anti-slip agents, plasticizers and biocides; substances that have been demonstrated can infiltrate from the plastic product to the sample and that can negatively influence the results of biological tests and laboratory measurements, thus offering a safe alternative for the preparation and storage of samples [26].

The data acquired by the aforementioned equipment were processed, applying the appropriate formulas for obtaining graphs that demonstrated the state of the protein after the conditions to which they were exposed, jointly measuring the concentration of the protein by absorption.

4.5 UV Absorption Spectroscopy Methodology

These measurements were performed on a JASCO model 7850 spectrophotometer, using a 1 cm cuvette of light passage. The parameters chosen were the following: 1 nm window, 0.2 nm data interval and 40 nm/min scan rate. Spectra were taken between 250 and 320 nm [25].

4.6 Circular Dichroism Methodology

These measurements were made on a JASCO J-810 spectropolarimeter calibrated with d-10 camphorsulfonic acid. Data in the near UV region (250-320 nm) or in the far UV region (200-250 nm) were collected at 25°C using quartz cuvettes of 10 or 1 mm pitch, respectively.

The molar ellipticity [θ] whose units are° cm^2 $dmol^{-1}$ was calculated according to the following formula:

$$[\theta] = \frac{100 \cdot \theta}{l \cdot c}$$

Where θ is the molar ellipticity in degrees (°) that is obtained from the experimental spectrum; l is the length of the passage of light in cm; c is equal to C / MWR, where C is the concentration of the protein in mg / ml (determined from the UV absorption spectrum) and MWR, the weight of the average amino acid residue for the protein, which is equal to Mr / N; where Mr is the molecular weight of the protein and N is the number of amino acid residues per protein molecule.

Typically, a scan rate of 20 nm/min and a time constant of 1 s were used. At least 3 consecutive spectra were taken from each sample, which were averaged to reduce signal noise [25].

4.7 Methodology of Thermal Denaturation

To evaluate possible changes in the conformational stability of the stored samples, both formulations were subjected to thermal denaturation. This was monitored following the drop in the ellipticity value at 222 nm. A volume of 2 ml of each of the protein solutions was heated from 25 to 90°C by means of a Peltier device at a speed of 1°C/min in a 1 cm quartz cuvette of optical passage sealed with a Teflon plug.

4.8 Fluorescence Methodology

Fluorescence measurements were made at 25°C on a Jasco FP-6500 spectrofluorimeter equipped with a thermostatted cell. For intrinsic fluorescence emission spectra, the excitation wavelength is 295 nm and the emission is collected in the 310-410 nm range. The bandwidth of the excitation and emission monochromators was set at 3 nm. A scanning speed of 100 nm min^{-1} was used with a time constant of 1 s. In all cases the corresponding targets were subtracted [25].

4.9 Methodology of Electrophoresis in Polyacrylamide Gels in the Presence of Sodium Dodecyl Sulfate (SDS-PAGE)

Electrophoresis in polyacrylamide gels in the presence of SDS was carried out as described by Schägger and Jagow [27]. The composition of the concentrating gel is 4% T, 3% C and that of the separating gel 16.5% T, 3% C.

The samples were heated for 3 min at 96°C in the presence of sample buffer (0.125 M Tris-HCl pH 6.8 , bromophenol blue 0.005% (w/v),SDS 4% (w/v), glycerol 20% (v/v), β-mercaptoethanol 2% (v/v)). The gels were prepared using Höefer's Mini-Prot equipment. A voltage of 70 V was applied during the entry of the sample to the gel and then the voltage was maintained at 100 V during the rest of the electrophoretic run.

The gels were stained with Coomassie Brilliant Blue G colloidal (CBB 0.1% w/v, $H_3(PO_4)$ 2% v/v, $(NH_4)_2SO_4$ 15% w/v) and stained with water [25].

4.10 HPLC Reverse Phase Methodology (RP-HPLC)

For the reverse phase chromatographic runs, we used a Rainin Dynamax chromatograph equipped with a C18 analytical column. Several circumvention schemes were raised by varying the relative concentration of solvents A (water, TFA 0.05%) and B (acetonitrile, TFA 0.05%) from 0 to 100%. A flow of 1 ml/min was used, monitoring the eluate absorption at 215 nm.

4.11 Dialysis

In this process a semipermeable dialysis membrane (molecular weight cut off 14 kDa) was used within which the sample to be dialyzed is placed, closed and subsequently placed inside a specimen with ~ 300 ml of buffer, kept under constant agitation overnight at 4°C.

4.12 Sterility

The plastic material was sterilized by autoclaving (at 121°C for 15 min).

Before analysis, the samples were filtered through a membrane with a porosity of 0.22 µm inside a laminar flow cabinet, to preserve sterility.

As a final control to verify sterility, LB agar plates (Luria Bertani) of the samples incubated 1 month at the set temperatures (4°C, 25°C, 37°C) were seeded. The plates were incubated at 37°C for 16 h.

5. RESULTS

In the present work were evaluated by UV absorption spectroscopy, fluorescence and circular dichroism, PF samples stored in two formulation buffers. The difference between them is in a single component: FO - the original buffer - contains sorbitol and FA - the alternative buffer - mannitol.

5.1 Effect of Dilution in FO and FA

Originally the samples are stored in prefilled syringes of PF (6 mg/ml) in FO. The first control that was carried out was to dilute the original sample in the formulation buffers sent by the laboratory.

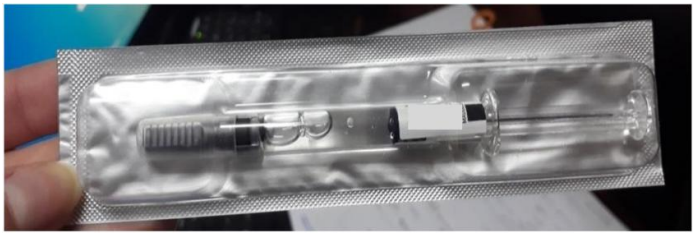


Fig. 5. One of the pre-filled syringes of pegfilgrastim used for the experiments

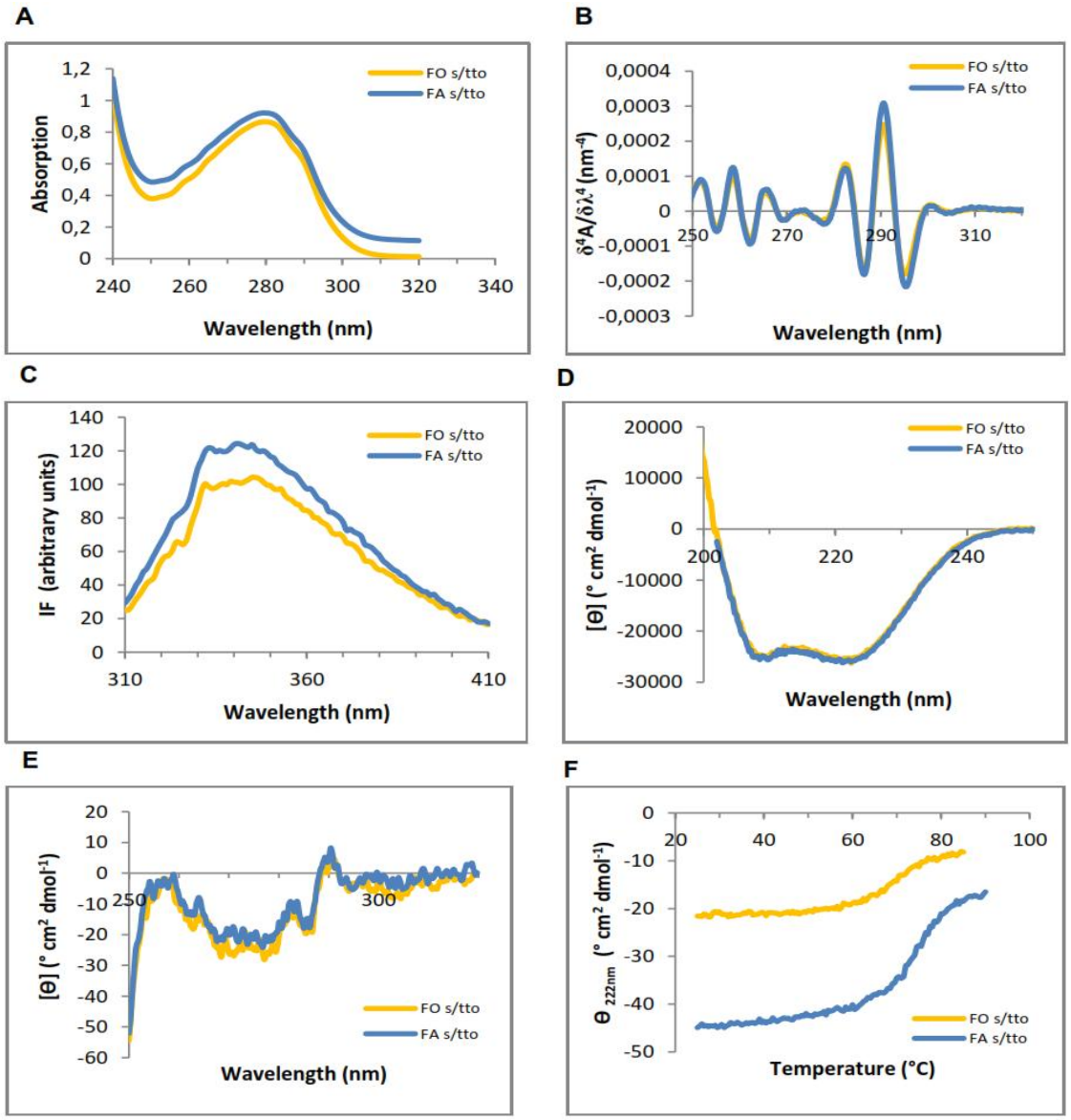
In Fig. 6, panel A shows the absorption of PF diluted in FO and FA buffer: FO and FA without any treatment (s/tto). Both spectra have a maximum absorption around 280 nm and a shoulder centered at ~ 290 nm, the latter being a characteristic of proteins that contain tryptophan residues in their sequence. Since the absorption value at 320 nm and at longer wavelengths is very low, we can rule out the presence of suspended aggregates.

Panel B shows the fourth derivative of the spectra shown above, thus we can notice that the curves are practically superimposable in intensity and that the maximums and minimums are located in the same position. The aforementioned indicates the conservation of the environment of the aromatic amino acids of PF in both formulations.

The fluorescence spectra obtained by diluting the protein in both formulations are plotted in panel C. The coincidence in the value of the wavelength corresponding to the maximum fluorescence emission

of tryptophan residues (λmax = 337 nm) indicates that they are in an environment of similar hydrophobicity.

Next, we will analyze the spectra of circular dichroism. The one obtained in the far UV region corresponds to panel D. This region allows us to assess globally the possible changes in the secondary structure of the protein. The spectra are indistinguishable, and two minima centered at ~ 208 and ~ 222 nm can be found, typical of a protein with high α-helix content. Panel E shows the spectra obtained for the near UV region; its high similarity shows the high analogy in the environments of the aromatic amino acids that make up the protein and, consequently, in its tertiary structure.
Finally, conformational stability was evaluated through a thermal denaturation experiment. For this, the PF solution was gradually heated and the evolution of the ellipticity was monitored in one of the minimums evidenced in panel F (222 nm). Next, the value of Tm was estimated, which indicates the value of the temperature corresponding to the midpoint of the transition and will be used as an indicator of thermal stability. In order to improve the comparative visualization of the results, the denaturation data will be shown normalized (panel G).



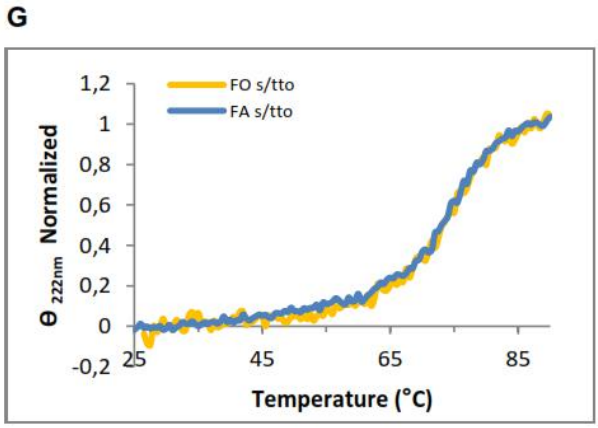


Fig. 6. Effect of dilution in FO and FA: UV absorption spectrum (A), fourth derivative of the spectrum shown in panel A (B), fluorescence spectrum (C), circular dichroism spectrum in the far UV regions (D) and close (E), thermal denaturation from 25°C to 90°C (F), thermal denaturation with standardized values θ_{222nm} for: PF at formulation pH (FO s/tto) and (FA s/tto)

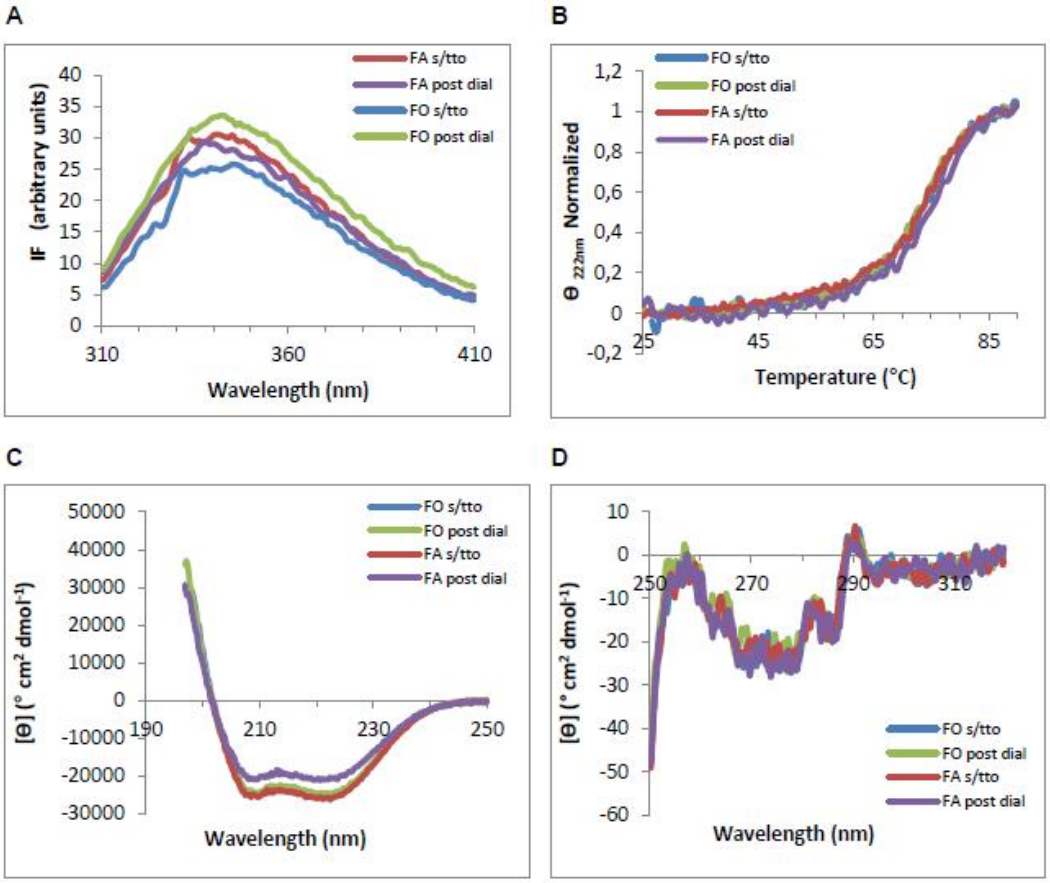


Fig. 7. Effect of dialysis: Fluorescence spectrum (A), thermal denaturation (B), circular dichroism spectra in far UV regions (C), near UV (D) of samples diluted in FO and FA buffer s/tto compared to FO and FA post-dialysis

In conclusion of this first control, we can affirm that the simple dilution in FO and FA buffer does not generate changes in the structure and conformational stability of the samples.

5.2 Effect of Dialysis

For the change of formulation to the alternative buffer, the samples were subjected to an O.N. at 4°C. As a control, the original sample was also dialyzed against FO buffer. They were then analyzed spectroscopically, and the results compared are shown in Fig. 7.

Despite the apparent modification in the secondary structure, the coincidence of the fluorescence spectra (λmax = 338 nm), in the CD in the near UV region and the value of Tm (~ 74.5°C), indicate that the structure and stability are not modified. This experiment allows us to rule out a possible deleterious effect caused by the mechanical stress in which the samples are subjected to being dialyzed. Since no changes were observed and in order to minimize the expense, handling and contamination of the samples, it was decided not to dialyze the sample that was stored in FO buffer. Therefore, it was only dialyzed against FA buffer.

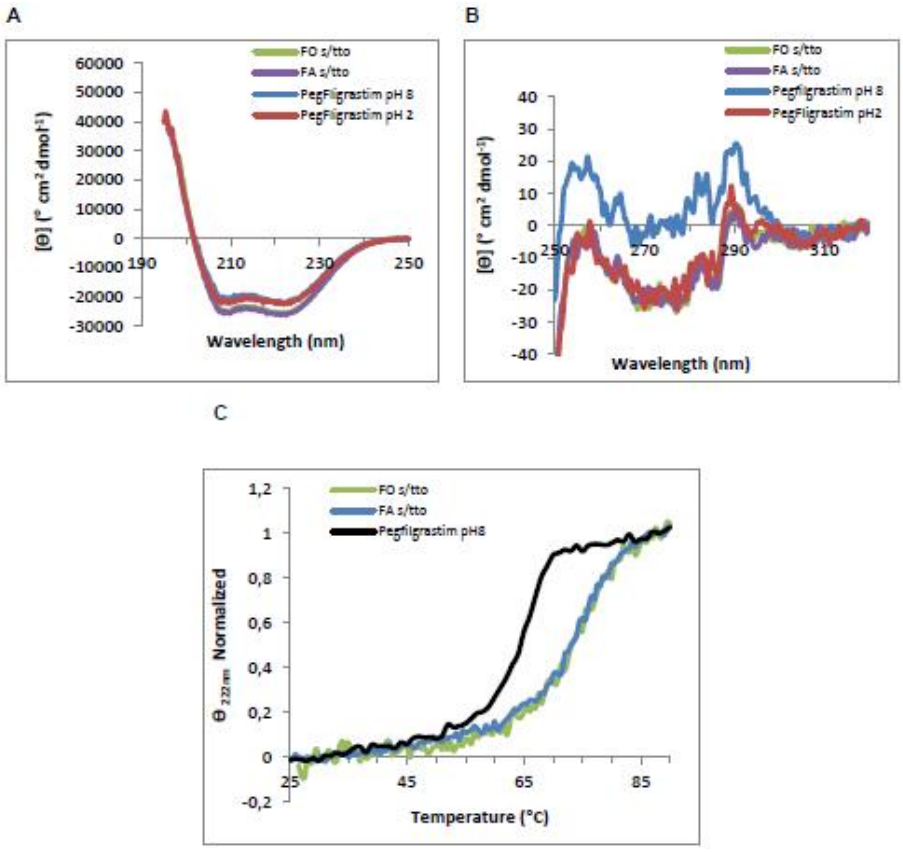


Fig. 8. Effect of pH change: Circular dichroism spectrum in the far UV region (A), near UV region (B) of the FO and FA s/tto samples compared to PF at pH 2.0 and 8.0; Thermal denaturation (C) of the FO and FA s/tto samples compared to PF at pH 8.0 (normalized by concentration)

5.3 Effect of Change in pH

Although the pH does not vary between both formulations, we decided to evaluate the impact of its modification. For storage, a pH of ~ 3.5-4.5 is chosen and we, by comparison, perform the

measurements at pHs away from it (2.0 and 8.0). The objective of this experimental approach was to look for a condition that shows changes in the structure and/or stability of the PF. Fig. 8 shows the results obtained.

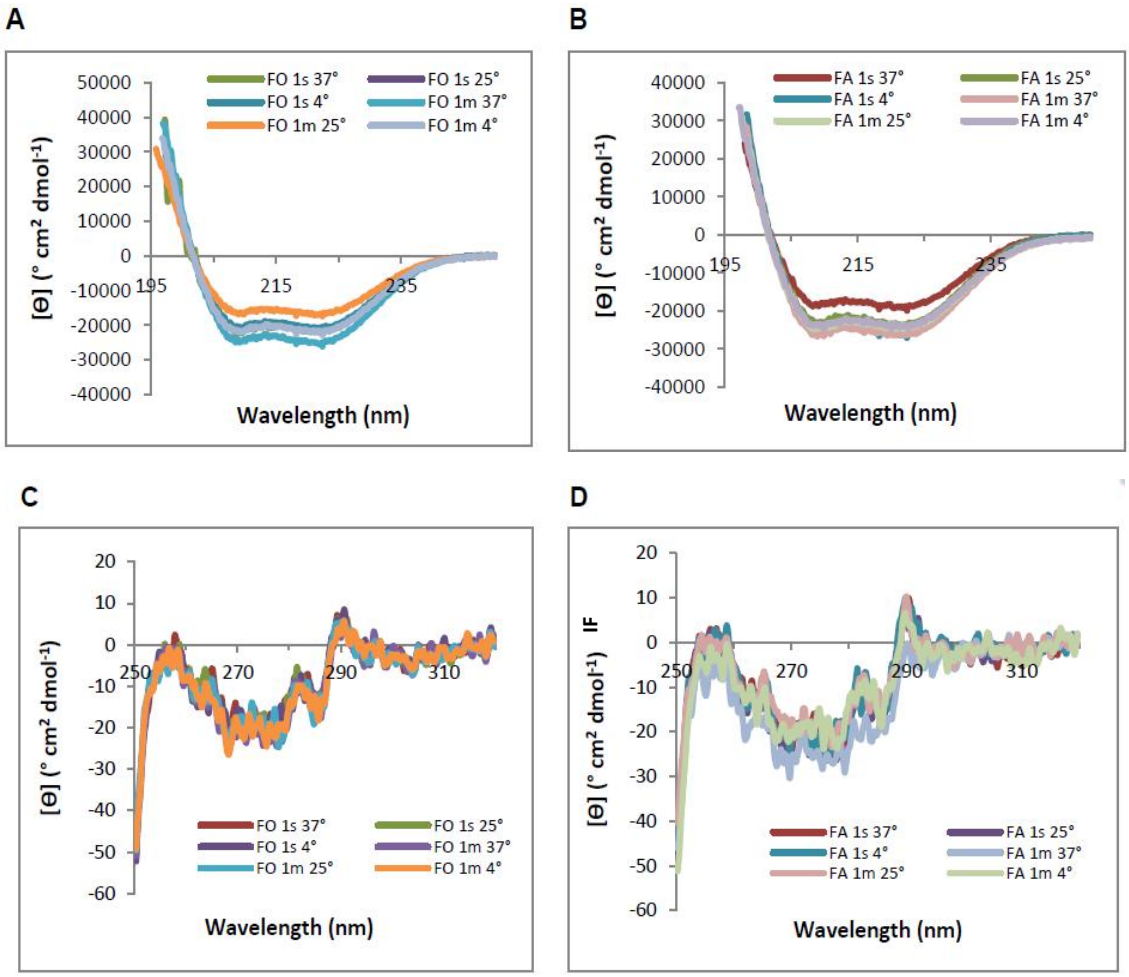
The results show that both PF at pH 2.0 and FO and FA s/tto maintain very similar structures in the far and near UV region; while in PF at pH 8.0 a marked difference in tertiary structure is observed (Fig. 8 B) compared to the other samples. Likewise, the difference is notable in the thermal ramp, whose Tm is significantly lower (~ 64.5°C), which indicates conformational instability at that pH.

This type of results shows a context in which the pH value selection criteria can be understood when designing the formulation.

5.4 Effect of Storage at Different Temperatures

The samples of PF in FO and FA buffer were aliquoted (200 µl) and stored in eppendorf of 200 µl maximum capacity. This was so because it is important to minimize the layer of air remaining on the sample in order to avoid possible oxidation during storage.

The samples were stored in a refrigerator (4°C), at room temperature (25°C), and in an oven (37°C), for one week and one month. The graphs of each analysis performed for both formulations are shown below in Fig. 9: original and alternative (In Annexes 2, 3 and 4 you can see all the graphs of the analyzes of each of samples stored at each temperature for one week and one month).



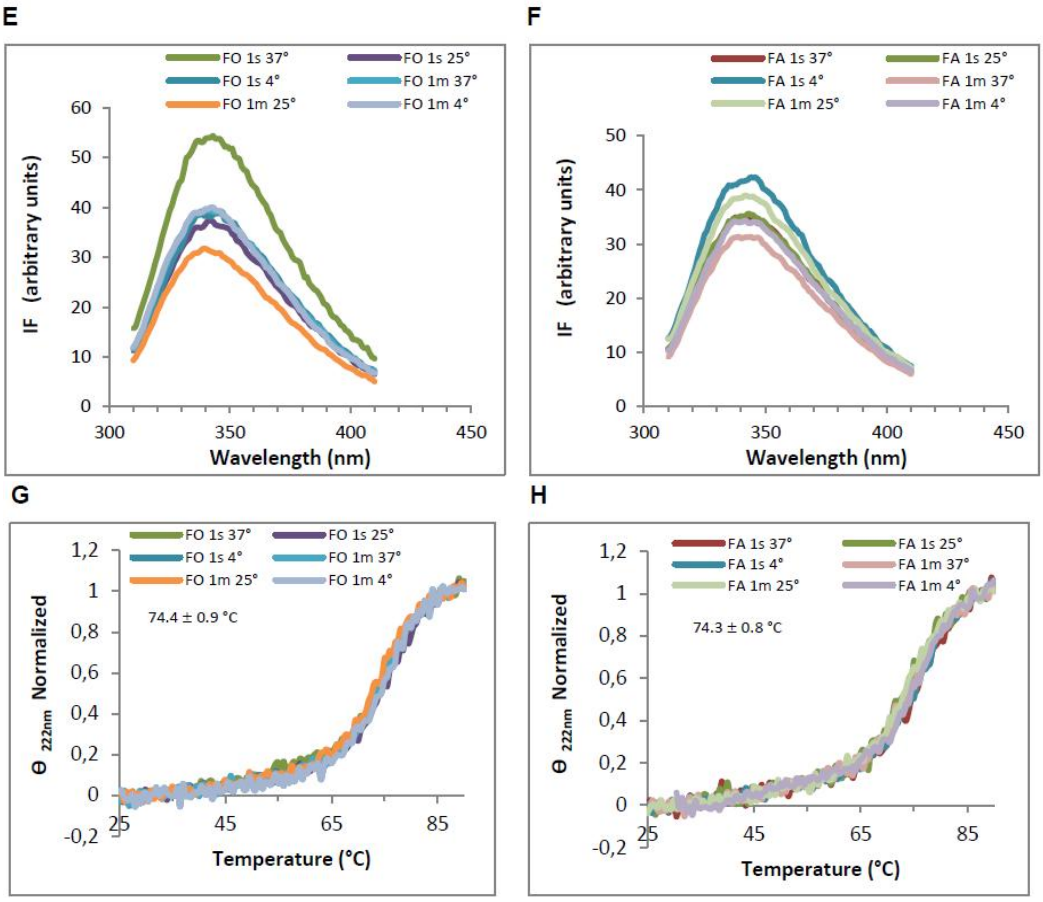


Fig. 9. Storage effect at different temperatures (4°C, 25°C, 37°C): Circular dichroism spectrum in the far UV region of FO samples (A), FA samples (B), UV region near FO (C), FA (D) samples, fluorescence spectrum of FO (E) samples, FA (F) samples, thermal denaturation of FO (G) samples, FA (H) samples all panels expose the Samples stored for a week and a month

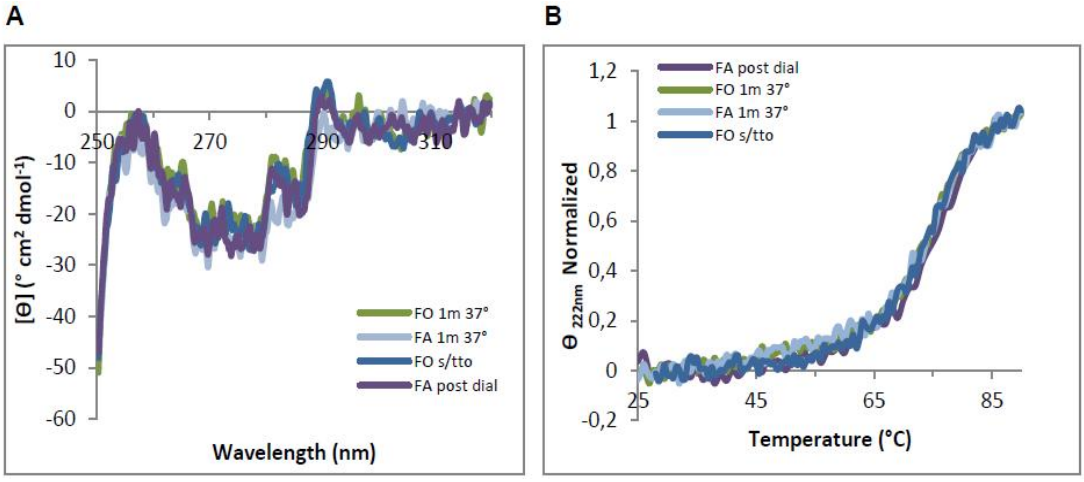


Fig. 10. Samples of FO and FA at 37°C stored for one month vs FO s/tto and post-dialysis FA. Circular dichroism spectrum in the near UV region (A), thermal denaturation (B)

In Fig. 9 it is shown that the samples stored at 4°C, 25°C and 37°C for one week and one month respectively, do not show major differences compared to the sample diluted in FO and FA buffer s/tto. However, in Fig. 10 the samples are observed at 37°C, which when stored for a month showed a small variation with respect to the others; however, when subjected to thermal denaturation, it shows a Tm ~ 74.5°C, the same value reflected in the other samples.

5.5 Effect of Extreme Temperatures

Alternatively, samples in both formulation buffers were subjected to storage at extreme temperatures. Freezing temperatures (-20°C and -80°C for one month) were explored on the one hand as well as an elevated temperature (57°C for 1 week). It was chosen at 57°C because at that temperature the beginning of the transition in the thermal denaturation curve would be marked.

In these cases, only thermal stability was evaluated (Fig. 11).

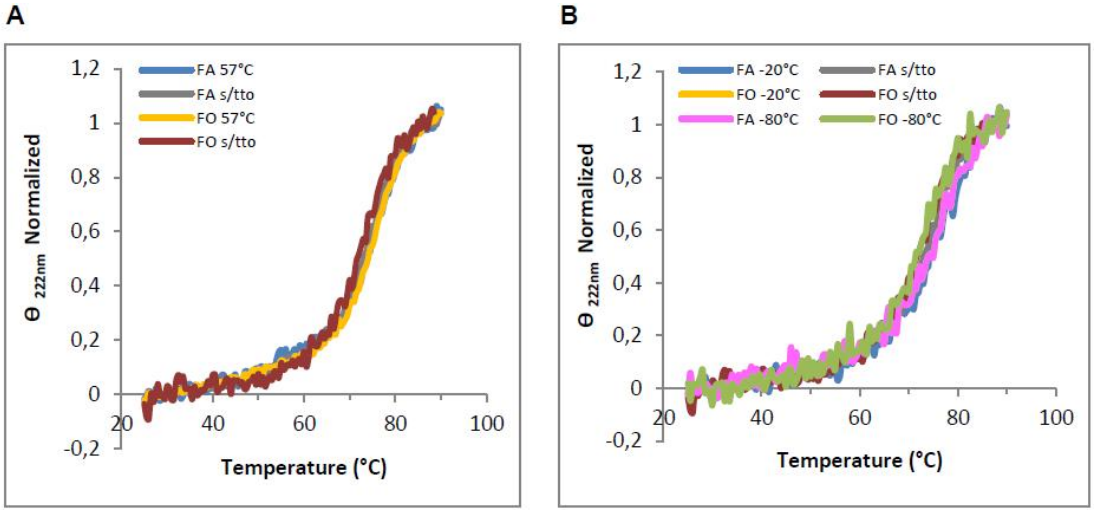


Fig. 11. Effect of extreme temperatures: Thermal denaturation of samples incubated at 57°C for one week (A), stored at -20°C and -80°C for one month (B).

From these graphs we can say that the samples stored at 57°C, -20°C and -80°C do not show many variations in their thermal stability with respect to the samples diluted in FO and FA s/tto, which is an indication that these treatments did not lead to massive alterations in protein conformation

5.6 HPLC Analysis

Analytical runs were performed under different conditions at 25°C and it was observed that the PF is not retained in the column. The European Pharmacopoeia recommends chromatography at 65°C. Our hypothesis is that you need this temperature increase to increase your flexibility and expose hydrophobic regions that can interact with the column. To verify this, we evaluate the conformation at 65°C and 90°C (temperature at which the PF is denatured). Figs. 12 and 13 show the results obtained.

5.7 Microbiological Control

Finally, a microbiological analysis was also carried out on Luria Bertani plates of all the samples used throughout the test.

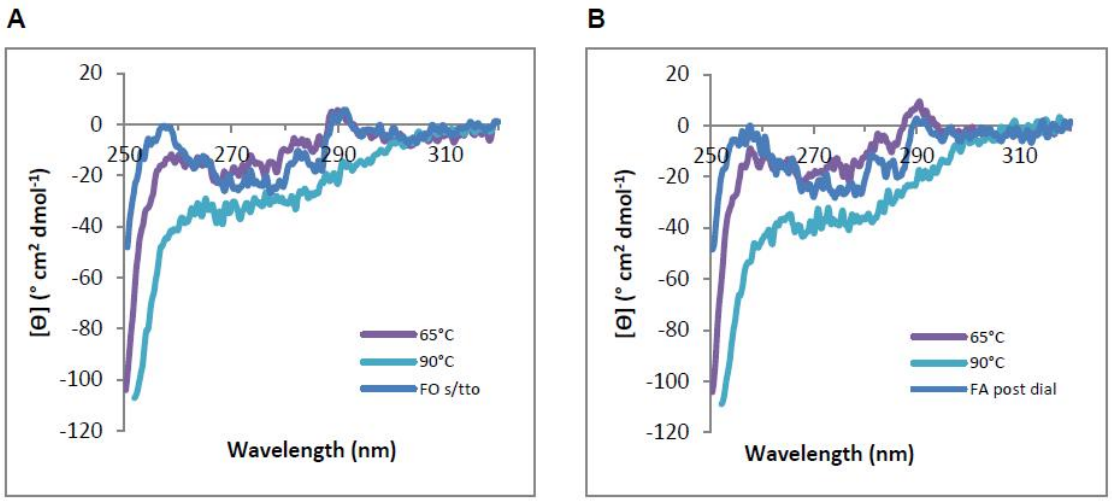


Fig. 12. A circular dichroism spectrum in the near UV region of FO samples stored at 65°C and 90°C (A), circular dichroism spectrum in the near UV region of samples at 65°C and 90°C (B). When comparing both panels a great difference can be noticed at 90°C, which undoubtedly reflects the loss of its tertiary protein structure

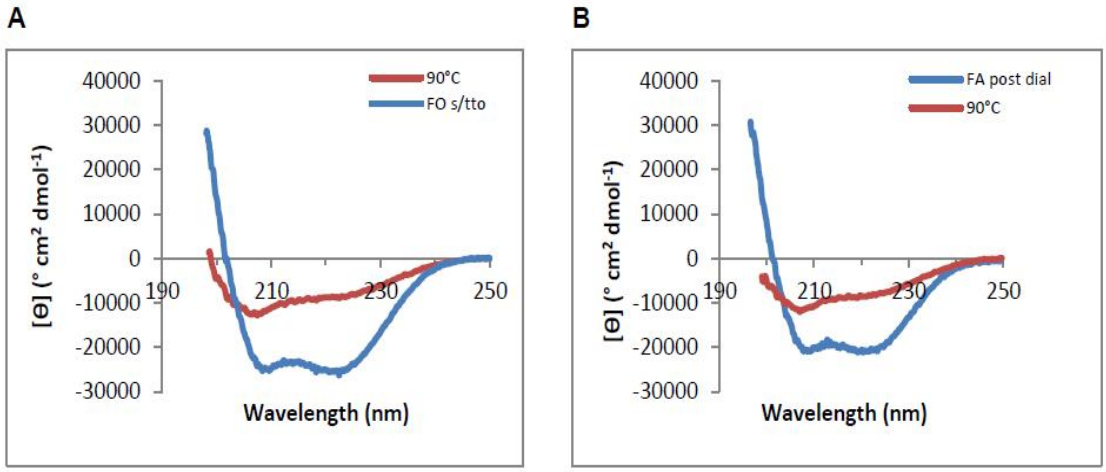


Fig. 13. Circular dichroism spectrum in the far UV region of FO sample at 90°C compared to FO without treatment (A). Spectrum in the far UV region of post-dialysis FA and FA at 90°C (B); both clearly demonstrate a loss of secondary structure at 90°C (denatured).

It can be seen that, in those samples in which there were colonies, stored at 37°C, were more susceptible to the growth of microorganisms, however; the other samples showed no growth, which indicates sterility.

5.8 Electrophoresis in Polyacrylamide Gels in the Presence of SDS (SDS-PAGE)

In this SDS-PAGE (stained with colloidal coomasie), the FO and FA samples were seeded at 3 storage temperatures and after 45 days of incubation. No fragments of low molecular weight are seen. Although no molecular weight markers were seeded, a 15 kDa protein with some proteolytic fragments of lower molecular weight was seeded on the last street.

Fig. 14. Microbiological control on LB agar of all FO and FA samples at the end of the experiment. 1) PF, 2) FO 37°C, 3) FA 37°C, 4) FO 25°C, 6) FA 25°C, 7) FO 4°C, 8) FA 4°C

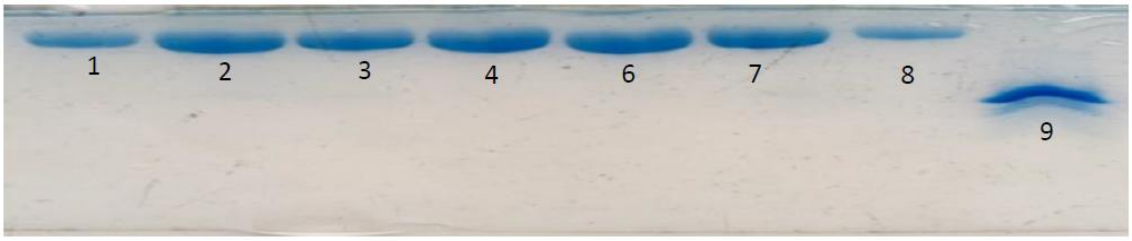


Fig. 15. Electrophoresis in polyacrylamide gels in the presence of SDS. ~ 3 µg of protein were seeded on each street: 1) PF, 2) FO 37°C, 3) FA 37°C, 4) FO 25°C, 6) FA 25°C, 7) FO 4°C, 8) FA 4°C, 9) a 15 kDa protein marker

6. DISCUSSION AND CONCLUSIONS

The development of a formulation includes the construction of a solid understanding of the physical and chemical degradation pathways of the molecule. Once these critical degradation pathways have been identified, robust tests capable of monitoring the degradants must be implemented. These tests will be used to verify that degradations are minimized under the expected storage conditions and that the formulation is robust enough.

Likewise, the proposed formulation should favor long-term protein stability under the expected storage conditions, as well as during manufacturing, shipping, storage, and administration. During these processes, the molecule may encounter potentially denaturing surfaces, undergo thermal stress for a short time or stress during freeze and thawing, and even experience agitation.

Piedmonte & Treuheit mention that mannitol is commonly used in lyophilized formulations because it easily crystallizes and provides a cake structure, so it is not ideal to apply it in a liquid formulation since the crystallization of the excipient in an accidental freeze can have a detrimental effect on protein stability. However, the analysis that were obtained, throughout the studied time, showed very similar results in terms of the secondary and tertiary structure of the protein dissolved in FO and in FA, despite the established temperature conditions at which the samples were exposed for one week and one month.

In this study the potential modification of the conformation and stability of the PF was evaluated due to a change of component in the formulation: Mannitol by sorbitol. Additionally, the samples were

subjected to mechanical stress due to dialysis and storage during different times and temperatures (-80, -20, 4, 25, 37 and 57°C).

The first control that was carried out was to dilute the original sample in the formulation buffers sent by the laboratory. Spectroscopic evidence demonstrated the similarity of the PF in both formulation buffers. The CD spectra obtained in the near UV region were indistinguishable. These have their minimums located at ~ 208 and 222 nm, which is typical for proteins with high α-helix content. Regarding its tertiary structure, the near UV region showed a high similarity in the environments of the aromatic amino acids that make up the protein. Finally, from the analysis of thermal denaturation - in which the PF solution was gradually heated - a similar Tm value was obtained in both samples, demonstrating comparable thermal stability.

The second control was the one in which the effect of dialysis was evaluated. Recall that this step was essential to change the original buffer for the alternative. Both samples were analyzed spectroscopically and, again, we did not observe that this process generated a deleterious effect on the PF samples. Notably, it has conservation of the tertiary structure and the value of Tm.

Although the pH is not modified in both formulations (~ 3.5 - 4.5), this parameter is very important when designing and defining a formulation. Therefore, as a comparison, measurements were taken at pHs away from it (2.0 and 8.0). The results showed that PF at pH 2.0 in FO and FA s/tto maintain very similar structures in the far and near UV region; while the PF at pH 8.0 marked a difference in its tertiary structure is observed in addition to a marked difference in the value of Tm obtained from thermal denaturation. At pH 8.0 the value obtained was ~ 10°C lower, which is indicative of a conformational instability. This emphasizes the importance of pH selection when formulating a biopharmaceutical product.

Samples that were stored in a refrigerator (4°C), at room temperature (25°C), and in an oven (37°C), both for one week and one month, would not show greater variations when compared to fresh samples diluted in FO and FA buffer (s/tto). Similarly, samples in both buffers that were subjected to storage at extreme freezing temperatures (-20°C and -80°C for one month) and at an elevated temperature (57°C for a week) are also comparable with those obtained by simple dilution in FO and FA buffer. The latter is a strong indication that, despite the different treatments to which PF was subjected, it regains its conformation and maintains its thermal stability in both formulations.

Finally, although we have not studied the effect of these treatments on degradation, oxidation, etc., in view of the spectroscopic results summarized here, we can say that the formulation with mannitol proved to be comparable with the original in terms of structural conservation of the PF, which constitutes it in a feasible candidate of being considered as an alternative for liquid formulations.

As a final reflection, the stability demonstrated by PF in both formulations against storage at 25°C, 37°C and even 57°C would give rise to the beginning of an exhaustive study that allows evaluating the feasibility of eliminating the cold chain in the transport and storage of these liquid formulations. It should be noted that, if possible, it would generate a great reduction in costs and, consequently, a decrease in the high sale price of these products.

7. SUMMARY

Filgrastim, a granulocyte colony stimulating factor (G-CSF), is a hematopoietic glycoprotein that binds with high affinity to its receptors in neutrophil precursor cells of the bone marrow thus, inducing proliferation and differentiation in neutrophils. The first-generation commercial product has a short half-life (between 3.5 to 3.8 hours). However, said half-life can be prolonged by means of a covalent modification with PEG (polyethylene glycol). Pegylation increases the hydrodynamic volume of the molecule, minimizing renal clearance. Thus, the use of pegylated molecule is more advantageous than the administration of several doses of "naked" protein.

Each pre-filled syringe of the original formulation contains 6 mg of pegfilgrastim in 0.6 ml of solution for injection, one of its components is sorbitol, a widely used tonicity modifier. In this work an

alternative formulation is proposed, replacing sorbitol with mannitol, which is mostly used in lyophilized formulations because it easily crystallizes.

In this study we propose to start the comparative evaluation of two formulations (FO: original and FA: alternative) of pegylated Filgrastim (PF) by means of a brief accelerated stability study focused on subjecting both formulations to different temperature and freeze-thaw conditions. Subsequently analyzing both its structure and its stability through different spectroscopies. The commercialization product and both formulation buffers were donated by a biopharmaceutical company. For the evaluation of the structural conformation in the established times (zero-time, one week and one month) both formulations were analyzed by UV spectroscopy, circular dichroism and fluorescence. Possible stability alterations were monitored by a thermal denaturation test.

Both formulations of PF were aliquoted and stored at 4°C, 25°C and 37°C. Another group of samples were kept at extreme temperatures (-80°C, -20°C -4°C and 57°C). At the time of carrying out the different measurements, the necessary dilutions were made in FO/FA. Regardless of the storage time and temperature, the samples formulated with mannitol or sorbitol showed no changes in the content of secondary structure or alterations in their tertiary structure. Also, we did not find alterations in its conformational stability. These preliminary studies show the viability of the replacement of mannitol by sorbitol in the PF formulation, since the analysis carried out through the proposed methods did not demonstrate protein instability linked to the change in formulation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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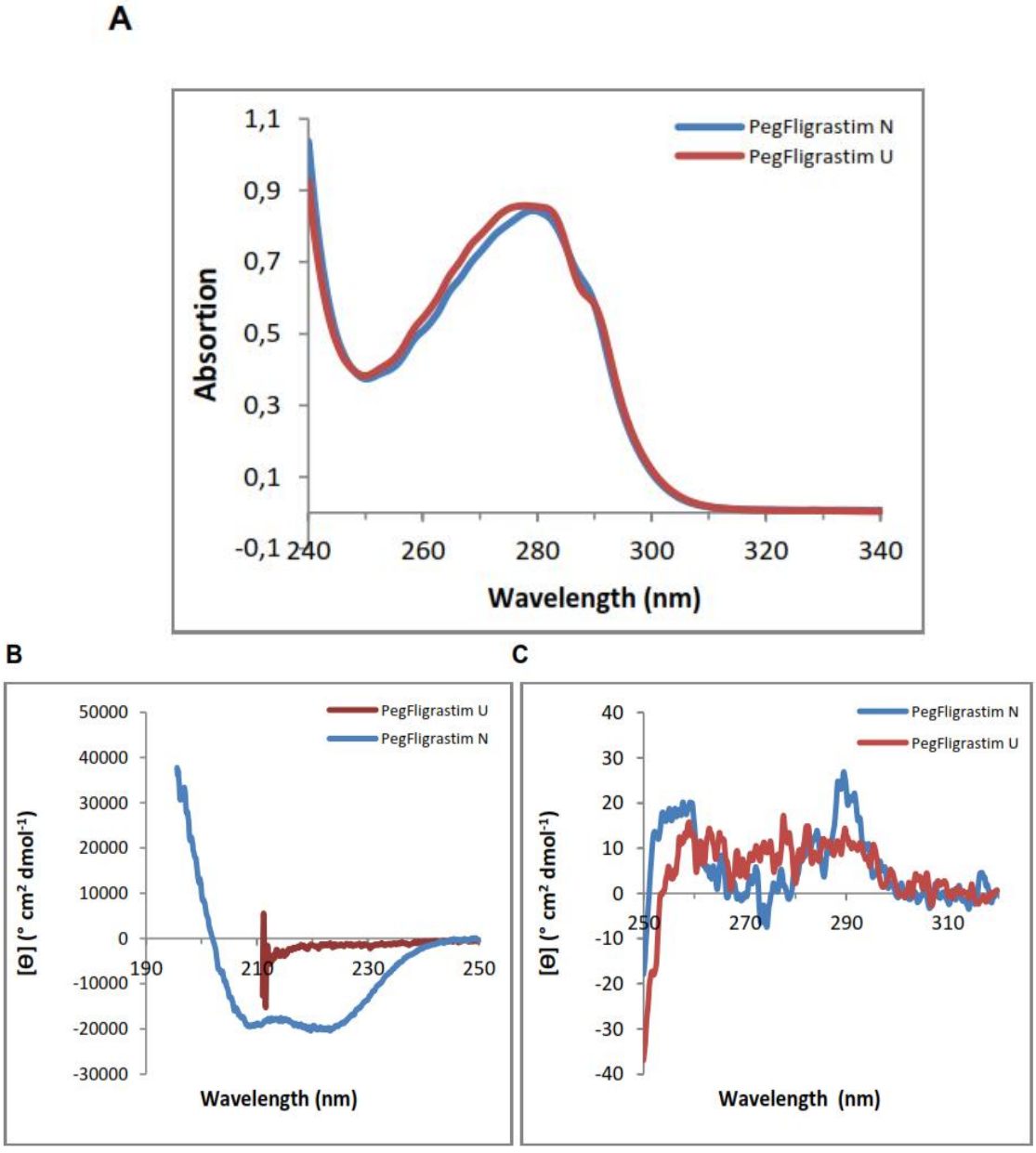
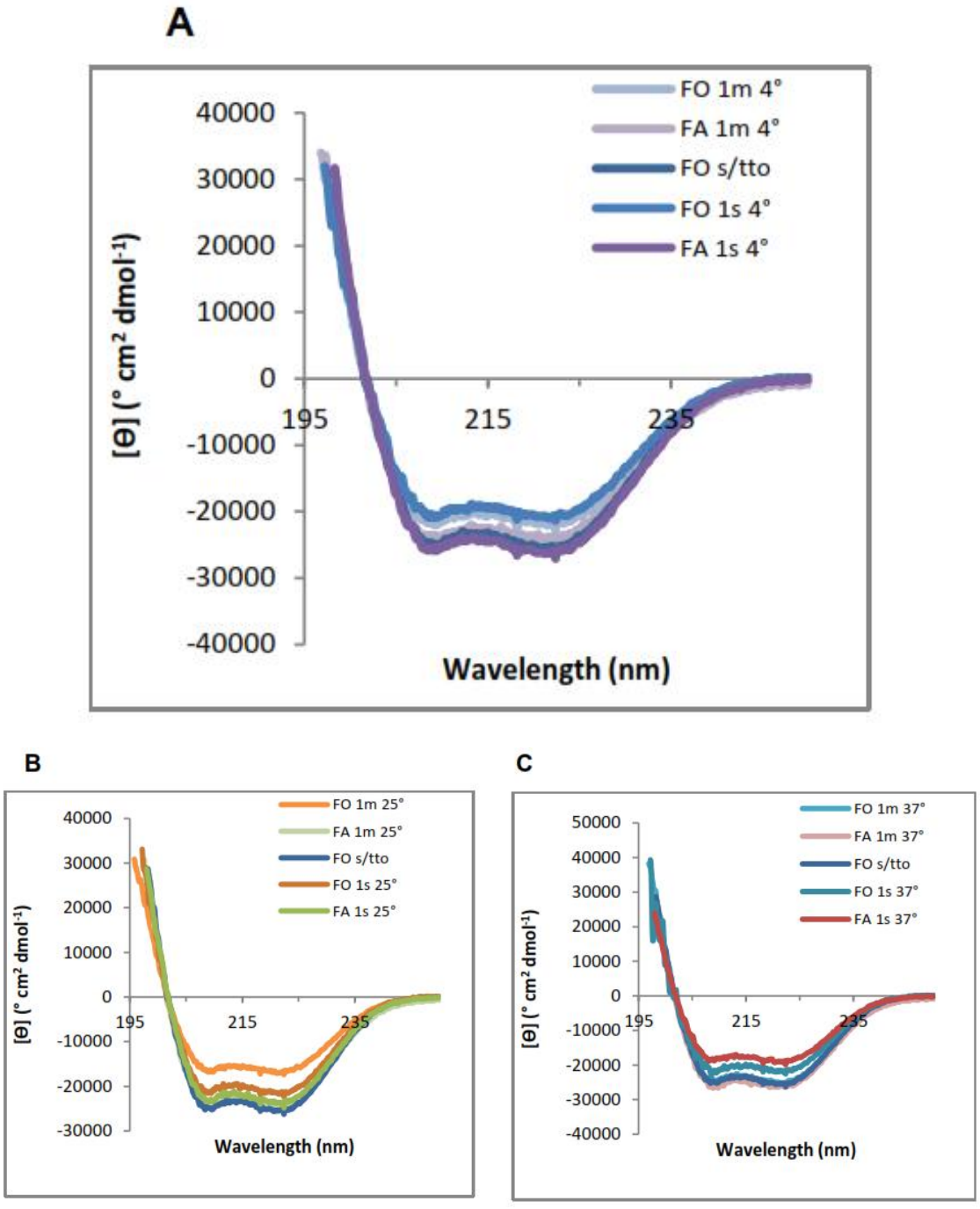
ANNEXES

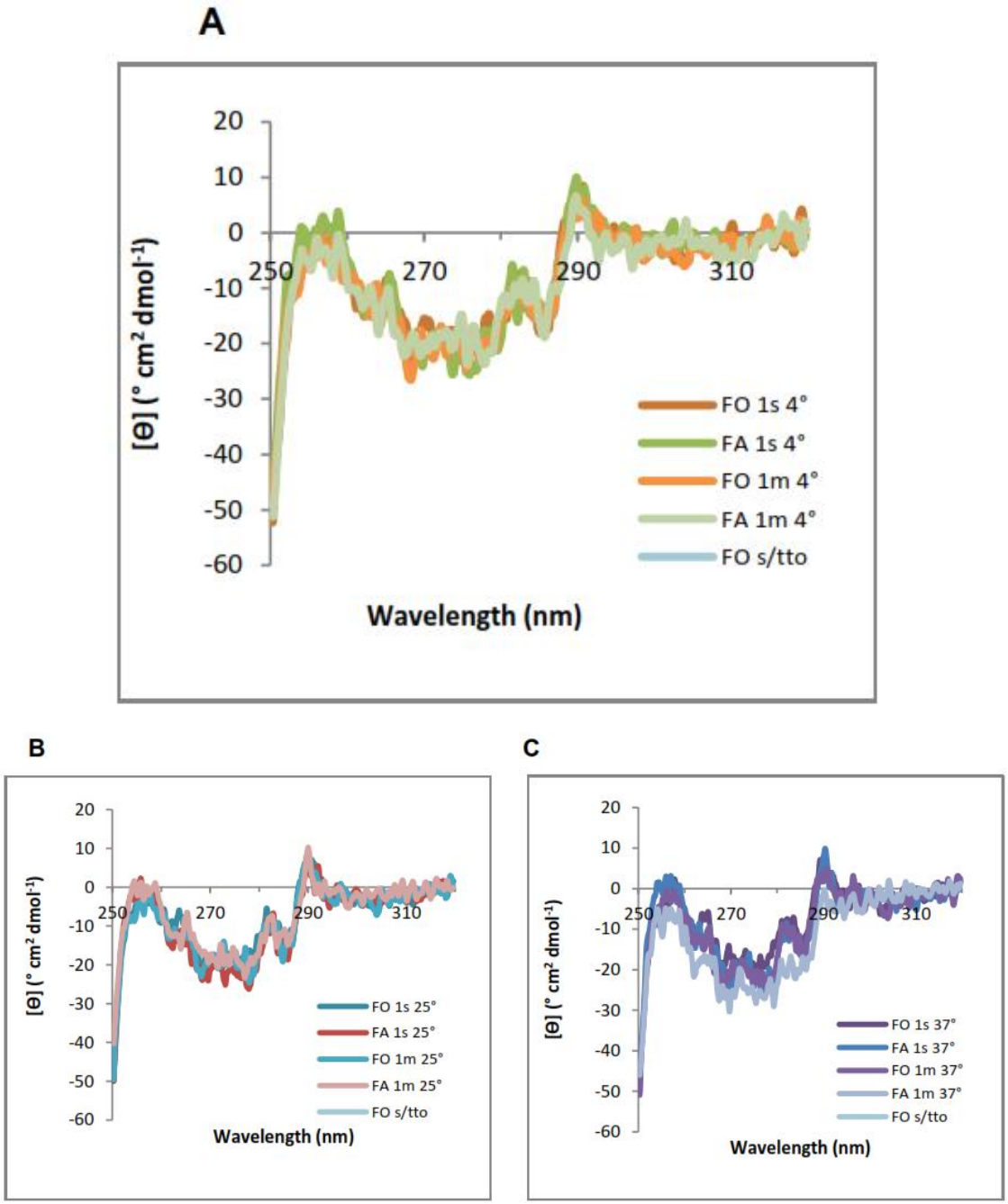
The data shown below highlights the differences in tertiary and secondary structure of native PF at pH 8.0 and denatured (in the presence of 7.1 M guanidinium chloride) at the same pH. In this way there is a reference of how a sample can be observed under such conditions.



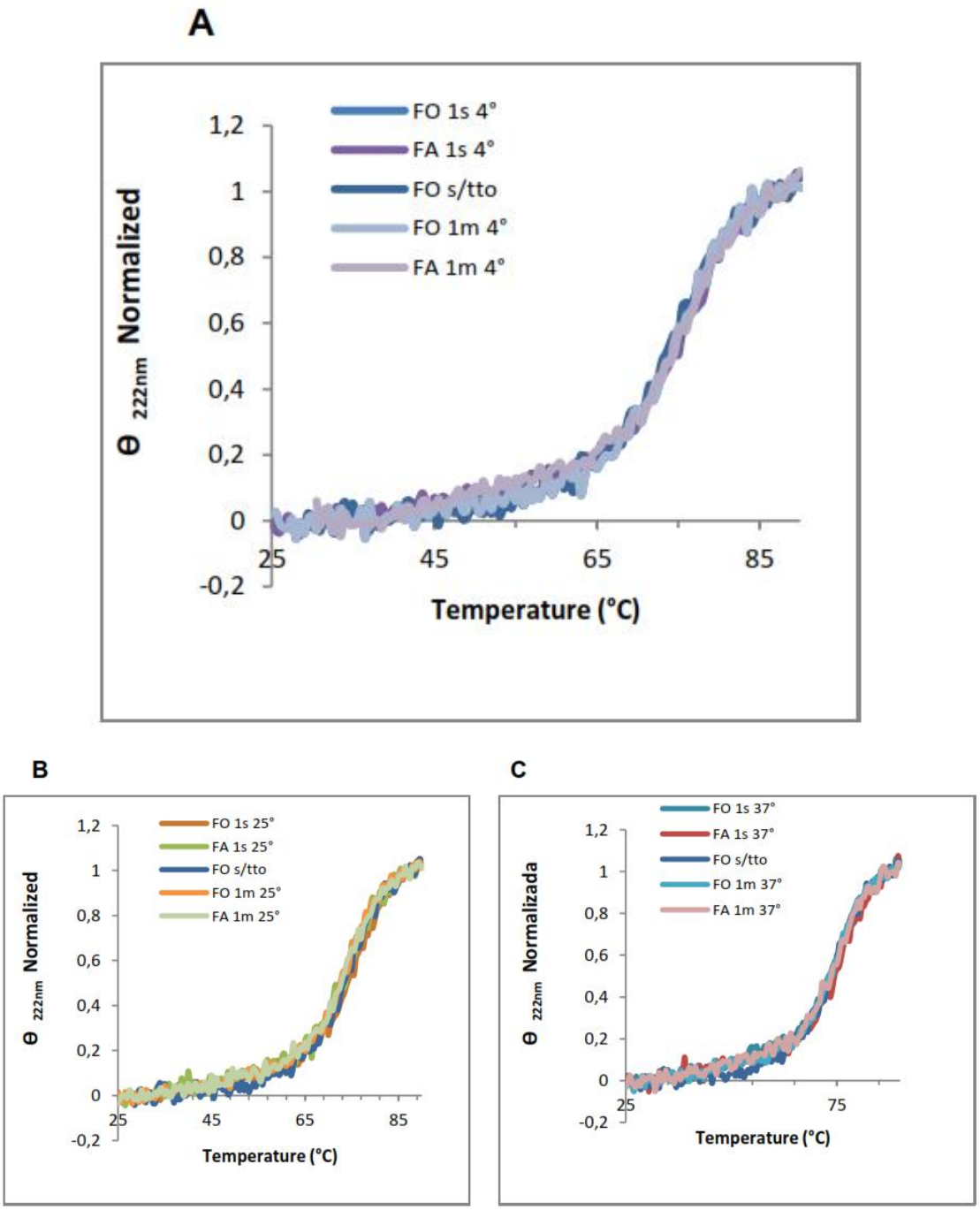
Annex 1. Panel A shows the absorption spectrum of native (N) and denatured (U) pegfilgrastim. panel B, the spectrum of circular dichroism in the far UV region, and panel (C), spectrum in the near UV region



Annex 2. Circular dichroism spectrum in the far UV region of FO and FA samples stored for one week and one month at 4°C (A), stored at 25°C (B), and 37°C (C)



Annex 3. Circular dichroism spectrum in the near UV region of FO and FA samples stored for one week and one month at 4°C (A), stored at 25°C (B), and 37°C (C)



Annex 4. Thermal denaturation of FO and FA samples stored for one week and one month at 4°C (A), stored at 25°C (B), and 37°C (C)

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Chapter 5
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Phamacognostical & Phamacutical Evaluation of *Ikshvaku Churna* [*Lagenaria vulgaris (Monila) Standly.* Fruit Pulp Powder]

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ABSTRACT

Lagenaria vulgaris (Monila) Standly.(Ikshvaku) is an annular herbaceous climbing plant with a long history of medicinal uses for treatment of various ailments including Jaundice, Diabetes, Ulcer, Piles, Colitis, Asthma, Insanity, Hypertension, CCf, Skin disorders. Its fruit pulp is used both as an emetic and purgative. Based on classical Ayurvedic textual indications and recent pharmacological studies its fruit pulp powder was selected for studying its emetic effect clinically on Bronchial Asthma. Before conducting the clinical trials, this fruit pulp powder was subjected to certain phamacognostical & Pharmaceutical studies to find the macroscopic & microscopic characters, pH, Ash value, Extractive values, Loss on drying, High performance Thin Layer Chromatography (HPTLC) etc. for standardization of the drug.

Keywords: Lagenaria vulgaris; Ikshvaku; pharmacognosy; phytochemestry.

1. INTRODUCTION

Herbal medicines are promising choice over modern synthetic drugs they show minimum or less side effects and considered to be safe. The earliest evidence of human use of plants for healing can date back to the Neanderthal period [1,2] According to WHO survey 80% of the populations living in developing countries rely almost exclusively on traditional medicine for their primary health care and needs [3]. There exists plethora of knowledge & information and benefits of herbal drugs in our ancient literature of Ayurvedic medicine, one of the earliest treatiser of Indian medicine; the Charaka Samhita (1000 B.C.) mentions the use of over 2000 herbs for medicinal purposes. Revival of interest in Ayurveda there has been a phenomenal increase in the demand for specialized therapies of Ayurveda out of which Panchakarma presents a unique approach of Ayurveda with specially designed five procedures of internal purification of body through the nearest possible roots. Such purification allows the biological system to return to homeostasis & to rejuvenate rapidly and also facilitates the desired pharmaco- theraputic effects of medicine administered thereafter [4]. Panchakarma has a full role of promotive, preventive and curative procedures. *Panchakarma* a specialty of *Kayachikitsa* (Internal Medicine) presents a unique approach of *Ayurveda* with specially designed five procedures of internal purification of the body through the nearest possible route [5,6]. Out of the five procedures of panchakarma Vamana or therapeutic emesis is the procedures of eliminating kapha dosha from the body through the upper passage by the vomiting. There are mainly six drugs i,e *catunaregam spinosa (Thunb)Tiruv-Madanphala, Luffa echinata Roxb.- Jeemutaka, Lagenaria vulgaris (Monila)standly-Ikshvaku, Luffa cylindrica (Linn.)-Dhamargava, Luffa acutangula (Linn) Roxb-Krutvedhana, Holarrhena antidysentrica (Roxb.ex Flem) wall-Kutaja* [7] recommended for therapeutic emesis in the form of different formulations in Charaka Kalpasthana [8]. Out of these drugs Madanphala is most commonly used for the process of emesis but there are certain drugs which are specifically indicated

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for treatment of particular diseases e.g. Dhamargava in Anemia, Ikshvaku in Diabetes, Asthma & Dry cough [9]. So it is a need of time to prove the pharmacological actions of these drugs on particular disease with the help of modern tools of standardization. Hence, to provide standard parameter for the quality control of Ikshvaku pulp powder in Bronchial Asthma, the present study was carried out.

1.1 Botanical Description [10]:

Lagenaria vulgaris (Monila)standley belonging to family cucurbitaceae, commonly known as Bitter bottle guard (Eng), Lauki (Hindi).It is a large pubescent, climbing or tailing herb with stout 5-angled hispid stems and bifid long tendrils. It found throughout the India either wild or cultivated. Leaves are long, petioled having 3-5 lobed, 7-10 or 10-12 cm. long. Fruits are 1-8 m large, bottle shaped with hard shell like epicarp when ripe. Seeds are numerous, long, white, smooth, 1.6-2 cm. long and horizontally compressed with marginal groove. Flowers are large white, solitary, monoecious. Seeds are many, obviate- oblong, white & compressed.

1.2 Aims and Objectives

Pharmacognostical, pharmaceutical and phytochemical analysis of *Lagenaria vulgaris (Monila) Standly* for setting a preliminary profile for further references.

2. MATERIALS & METHODS

2.1 Pharmacognostical Evaluation

Lagenaria vulgaris (Monila) standly fruits were collected from the local farms of Chandrapur district, Maharashtra in the month of December-January. The fresh and semi-ripped fruits were cut into small pieces. The pulp obtained from fruits were shade dried & made into the coarse powder later the organoleptic and powder microscopy of fruit pulp powder was carried out at pharmacognosy department of I.P.G.T. & R.A. GAU, Jamnagar.For microscopic observation The fruit pulp powder was treated with small quantity of distilled water on the slide, and then it is again stained with phloroglucinol & conc.HCl to find out the lignified materials along with other cellular constituents. The micro photographs were taken under Carl Zeiss Binocular microscope attached with camera.

2.2 Pharmaceutical and Phytochemical Evaluation

Lagenaria vulgaris (Monila) standly fruit pulp powder was analyzed using various standard physicochemical parameters such as loss on drying, ash value, water soluble extract, alcohol soluble extract and pH value [11]. In qualitative analysis presence of carbohydrate, phytosterol, saponin, tannins, flavonoids were assessed. HPTLC were carried out after making appropriate solvent system with Methanolic extract of *Lagenaria vulgaris (Monila) standly* fruit pulp powder at the Pharmaceutical chemistry lab, IPGT & RA.

Qualitative tests: The methanol extract of the sample was analyzed for different functional groups. The presence of carbohydrate, phytosterol, saponin, tannins and flavonoid were confirmed through suitable tests [Table 2].

HPTLC: Methanol extract of *Lagenaria vulgaris fruit pulp powder* was spotted on pre coated silica gel GF 60_{254} aluminum plates by means of Camang Linomat V sample applicator fitted with a 100 µL Hamilton syringe. Chloroform: MeOH (9:1) was used as the mobile phase. After development densitometric scan was performed with a Camag T. L. C. scanner III in reflectance absorbance mode at U.V. detection as 254 nm and 366 nm under control of Win CATS Software (V 1.2.1. Camag). After completion of chromatographic procedure spraying of the plate was done with Anisaldehyde and the spots obtained were observed in day light.

3. OBSERVATION AND RESULT

3.1 Pharmacognostical Analysis

Organoleptic characters:

Color- Light cream
Odour- Pungent
Taste- Bitter
Microscopic Observations:

Diagnostic characters under microscope are fibers (Fig. 1), fragments of annular vessels (Fig. 2), prismatic crystals of Calcium Oxalate (Fig. 3), tannin (Fig. 4), loosely arranged parenchymal cells (Fig. 5), simple starch grains (Fig. 6), spiral vessels (Fig. 7), pitted scleroid (Fig. 8), mesocarp cells (Fig. 9).

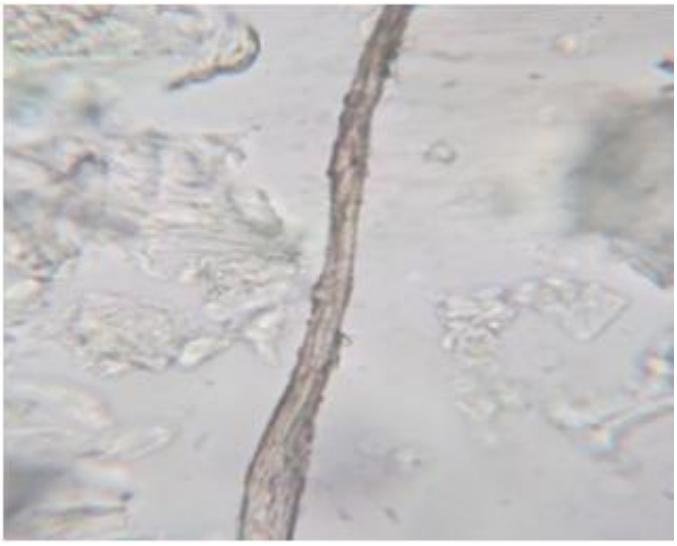


Fig.1. Fibre

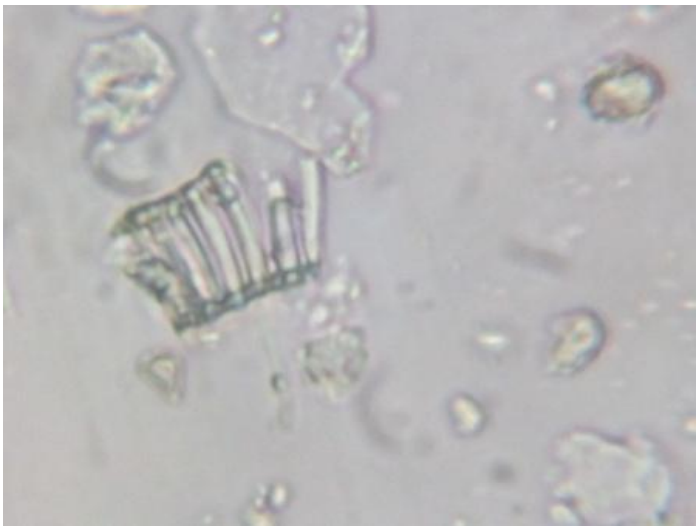


Fig. 2. Fragments of annular vessels

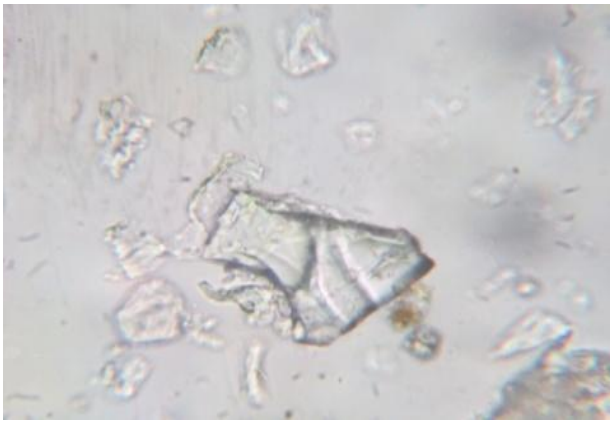


Fig. 3. Prismatic crystal of calcium oxylate

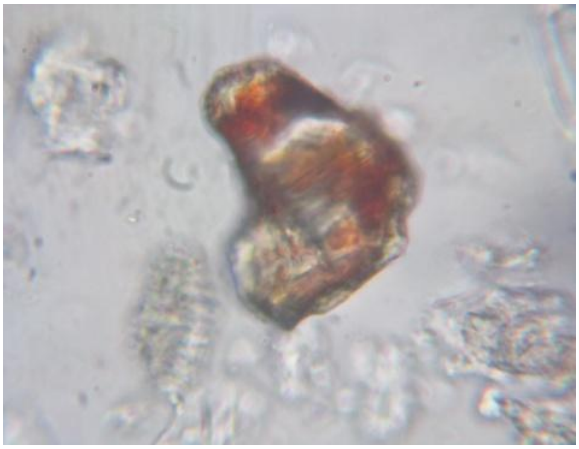


Fig. 4. Tannin content

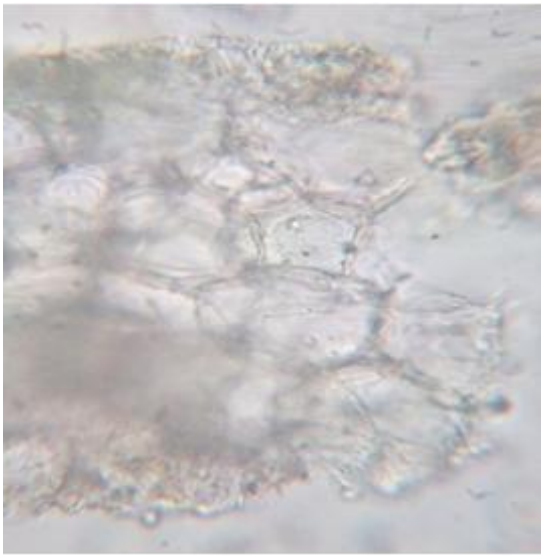


Fig. 5. Loosely arranged parenchyma cells

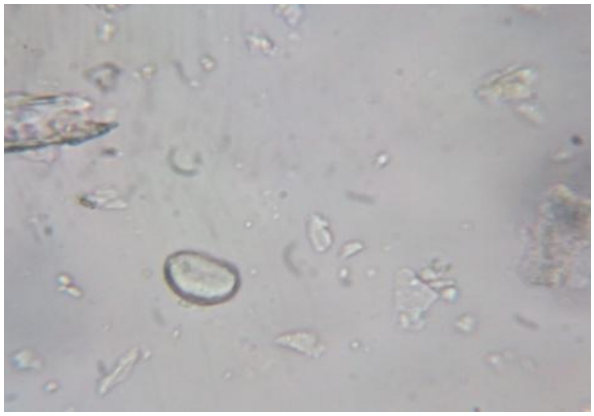


Fig. 6. Simple starch grain

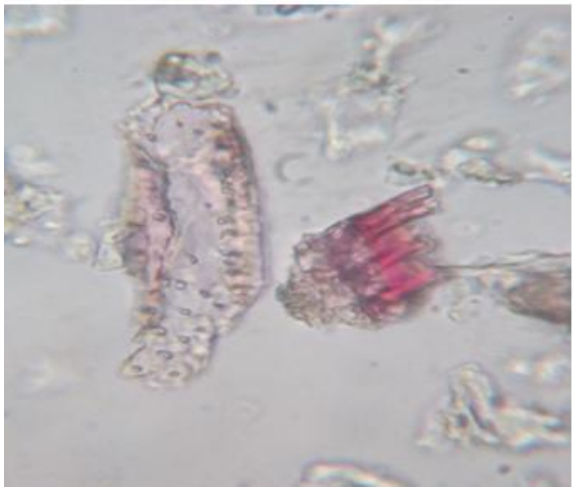


Fig. 7. Pitted scleroid

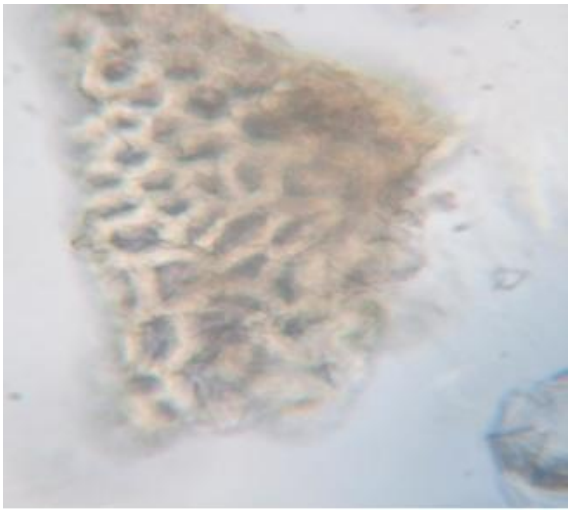


Fig. 8. Mesocarp cells

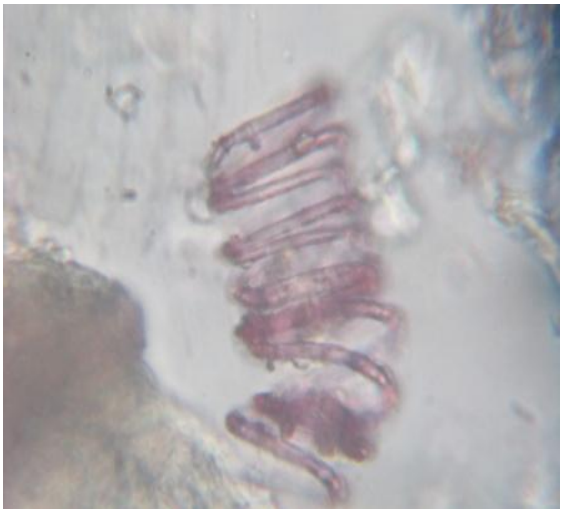


Fig. 9. Spiral vessels

3.2 Pharmacutical Analysis

Lagenaria vulgaris (Monila) standly fruit pulp powder was analyzed using various standard physicochemical parameters. All the Pharmaceutical parameters such as loss on drying, ash value water soluble extract, alcohol soluble extract and pH Value were analysed (Table 1.)

3.3 Phytochemical Analysis & HPTLC

Qualitative tests: Presence of glycosides, flavonoid and tannins were confirmed through the suitable tests (Table 2).

3.4 HPTLC

On analyzing under densitometer Track Showed 5 spots under 366 nm with Rf 0.01,0.12,0.20,0.50,0.90 and 9 spots were seen. Under 254nm with Rf 0.01, 0.09, 0.14, 0.18, 0.27, 0.50, 0.64, 0.73, 0.87 (Table 3).

Table 1. Physicochemical parameters

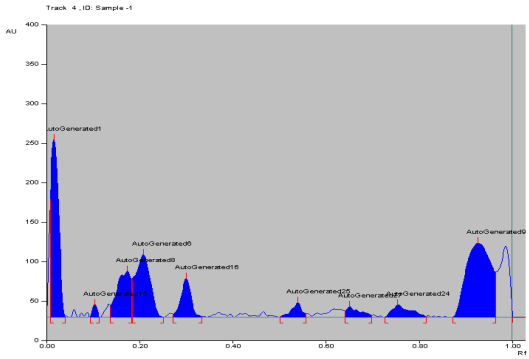
Sr.No.	Test	Result
1.	Aqueous Extractive	49.12 %w/w
2.	Alcohol Extractive	46 %w/w
3.	pH	6.0
4 .	Ash value	10.6304 % w/w
5.	Loss on drying	10.4669 % w/w

Table 2. Qualitative test

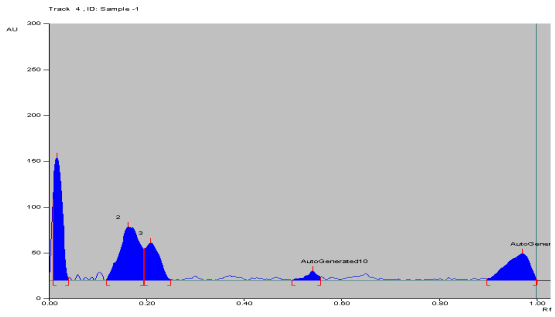
Material	Functional group	Result
Alcoholic Extract of fruit pulp powder of *Lagenaria vulgaris*	Phytosterol	Present
	Flavanoids	Present
	Tannins	Present
	Saponine	Present

Table 3. HPTLC

HPTLC	SPOTS	R_f Values at 254 nm
	9	0.01, 0.09, 0.14, 0.18, 0.27, 0.50, 0.64, 0.73, 0.87
		R_f Values at 366 nm
	5	0.01, 0.12, 0.20, 0.50, 0.90.

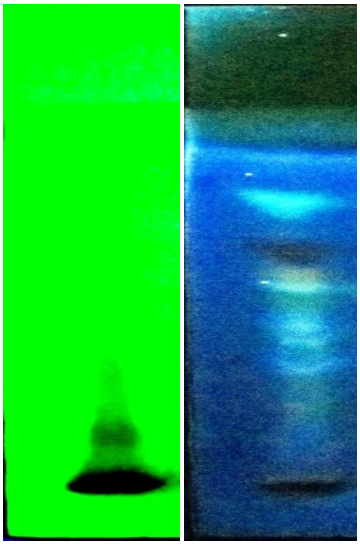


Densitogram curve of methnol extract of *Lagenaria vulgaris* fruit pulp powder at 254 nm



Densitogram curve of methnol extract of *Lagenaria vulgaris* fruit pulp powder at 366 nm

Fig. 10. Densitogram of *Lagenaria vulgaris* fruit pulp powder



254 nm **366 nm**

Fig. 11. HPTLC of *Lagenaria vulgaris* fruit pulp powder

4. DISCUSSION

Pharmacognocy study helps in authentication of the commonly used drugs through morphological, histological and physico-chemical parameters. This can prevent the accidental misuse of drugs and adulteration to a greater extent. In the present study the sample was proved to be genuine by assessing the pharmacognostical Parameters. Evaluation of physico-chemical parameters and

qualitative analysis helps to identify the presence of specific ingredients in a sample and application of chromatographic techniques aid in recognition of number of ingredients and also to assess the purity by comparing with the standard ones. Refractive index procedure was not applicable to the *churna* so the same could not be assessed. pH is the measure of acidity or basicity of a solution. In the present sample pH was detected by using pH indicator paper and it was 6 showing the alkaline nature of the solution. Loss on drying method is applied to determine the amount of water, all or a part of water for crystallization, or volatile matter in the sample. Loss on drying of test drug is 10.4669% w/w. Total ashes are designed to measure the total amount of material remaining after ignition. It includes both physiological (which is derived from the plant tissue itself) and non-physiological ash (residue of the extraneous matter likes and etc adhering to the plant substance) Ash value of *Lagenaria vulgaris* fruit pulp powder is 10.6304% w/w. Water soluble extract & alcohol soluble extract is 49.12% w/w & 46% w/w respectively. Thin layer chromatography is the most common form of chromatographic method used by Ayurvedic research workers to detect the number of compounds present in a product. It also helps to determine the purity of the sample. Identity of a compound is also possible by comparing it with the Rf value of a known compound. Here for the purpose of conducting.

TLC tracks were made having the sample ethyl acetate extract of *Lagenaria vulgaris* fruit pulp powder. After careful analysis and discussion with experts the mobile phase was fixed to be Toluene+Ethylacetate in the proportion of 7:3 respectively. The sample tracks and mobile phase remained the same for all the experiments related to TLC .The spots produced by TLC were observed in day light , short UV and long UV and Rf value was calculated .Track showed 5 spots under 366nm with Rf 0.01,0.12,0.20,0.50,0.90 and 9 spots were seen under 254nm with Rf 0.01, 0.09, 0.14, 0.18, 0.27, 0.50, 0.64, 0.73, 0.87 After completion of chromatographic procedure spraying of the plate was done with Anisaldehyde and the spots obtained were observed in day light.

5. CONCLUSION

Identified phytochemical components content in the present sample i,e, tannins have highly antioxidant & anti-inflammatory function [12], saponin shows steroidal aglycone structure with antibacterial & adjuvant property [13], phytosterol promise in allergies and stress related illness [14], flavonoids are antioxidant, anti-inflammatory, anti-allergic relives hay fever, eczema, sinusitis [15]. All this support the intended action of the given sample in management of Bronchial Asthma. It is inferred that the given sample meets minimum qualitative standards as prescribed by API at preliminary level. The results of this study may be used as the reference standard in further research undertakings of its kind.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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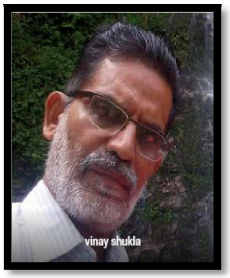

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Chapter 6
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Swelling and Drug Release Studies from Hydrophilic Matrices Containing Combination of Different Grades of Hydroxyl Propyl Methylcellulose

Masheer Ahmed Khan[1*] and S. C. Chaturvedi[1]

DOI: 10.9734/bpi/tprd/v5

ABSTRACT

Polymers are very popular and widely used in formulating sustained release tablets because they are excellent drug carriers. The current study examines the drug release from the hydrophilic matrices prepared using combination of different grades of hydroxyl propyl methylcellulose (HPMC), viz, HPMCK4M, HPMCK15M and HPMCK100M. The results indicate that swelling and release profiles were affected by concentration and viscosity grade of the polymer. This swelling action of HPMC of different grades in turn is controlled by the rate of water uptake into the matrices. An inverse relationship exists between the drug release rate and matrix-swelling rate. This implies that rational combination of the different grades tends to provide quite regulated release of drug. The swelling behavior is therefore useful in predicting drug release.

Keywords: Hydroxy propyl methy cellulose (HPMC); polymers; swelling; matrices; release rate.

1. INTRODUCTION

Hydrophillic matrices are the least complicated devices in the formulation of sustained release dosage form. Hydroxypropyl methyl cellulose (HPMC) of different grades are used as the gel forming agent in matrices [1-2]. Swellable systems consisting of hydrophilic polymers, in the presence of water, absorb a significant amount of water dissolution medium to form gel. As the dissolution medium penetrates the matrix, polymer material swelling starts and drug molecules begin to move out of the system by diffusion [3-8].

The aim of the present study was to investigate the role of combination of different grades of polymer on swelling, the effect of swelling on drug release and also to describe the release kinetics. Drug release data from HPMC matrices follows the classical Higuchi dissolution equation relating drug release with square root of time [9-12].

2. MATERIALS AND METHODS

Zolpidem tartrate was obtained as a gift sample from Ranbaxy Labs. Ltd, Dewas, (M.P.), Methocel (K4M, K15M, K100M) were provided by Colorcon India Ltd., Goa, dicalcium phosphate, microcrystalline cellulose (Avicel pH_{101}), talc, magnesium stearate and all other reagent used were of analytical grade.

2.1 Preparation of Matrices

Nine formulations employed for investigations containing different ratios of HPMC of different grades were prepared by direct compression and coded C1, C2, C3, D1, D2, D3, E1,E2 and E3. The ratios of different grades of HPMC employed are shown in Table 1. The amount of drug, magnesium stearate, MCC and talc were kept constant while dicalcium phosphate was taken in sufficient quantity to maintain a constant tablet weight of 120 mg. All the products and process variables (other than the

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concentrations of two polymers) like mixing time, compaction force, etc, were kept constant. Ten tablets from each batch were weighed individually and subjected to physical evaluation.

Table 1. Different ratios employed in formulations containing HPMC of different grades

Formulation Code	HPMC K_4M	HPMC $K_{100}M$	ZPM	Formulation Code	HPMC K_4M	HPMC $K_{15}M$	ZPM	Formulation Code	HPMC $K_{15}M$	HPMC $K_{100}M$	ZPM
C1	1	1	1	D1	1	1	1	E1	1	1	1
C2	2	2	1	D2	2	2	1	E2	2	2	1
C3	3	3	1	D3	3	3	1	E3	3	3	1

2.2 Matrix Swelling and Water Uptake Studies

Swelling was evaluated by weight. The matrices were placed in 900 ml dissolution medium pH 6.3, at 37°C. At different time intervals, the previously weighed tablets were removed, gently wiped with a tissue to remove surface water, and reweighed. The percent water uptake i.e., degree of swelling due to absorbed test liquid, can be estimated at regular time intervals using the following equation –

$$\% \text{ water Uptake} = (Ws-Wi)/Wp *100$$

Where, Ws = Wt. of the swollen matrix at time t, Wi = Initial wt. of the matrix, Wp = wt. of the polymer in the matrix. The polymer swelling or water uptake are mean of three determinations. The degree of swelling can be calculated by the following formula –

$$\text{Degree of swelling} = Ws-Wd/Wd*100$$

Where,Wd = Final dry wt. of the matrix, Ws = Swollen wt. of the same matrix at immersion time (t), The swelling degree is the mean of at least three determinations.

2.3 Dissolution Studies

Dissolution studies were carried out for all the nine formulations in triplicate, employing dissolution apparatus, using distilled water pH 6.3 as the dissolution medium at 50 rpm and 37 $\pm$ 0.5^0C. An aliquot of sample was periodically withdrawn at suitable time intervals and volume replaced with equivalent amounts of plain dissolution medium. The samples were analyzed at 245 nm.

3. RESULTS AND DISCUSSION

The weight of the polymer in the matrix (Wp) and final dry weight of the matrix (Wd) are shown in Table 2. The weight of the swollen matrix at different time intervals, degree of swelling and percent water uptake data was observed.

Table 2. Final dry weight and weight of polymer in matrix tablets of different batches

Formulation Code	Final dry weight (Wd)(mg)	Weight of polymer in matrix (Wp) (mg)	Formulation Code	Final dry weight (Wd) (mg)	Weight of polymer in matrix (Wp) (mg)	Formulation Code	Final dry weight (Wd) (mg)	Weight of polymer in matrix (Wp) (mg)
C1	120	24	D1	121	24	E1	124	24
C2	127	48	D2	120	48	E2	125	48
C3	125	72	D3	124	72	E3	122	72

The results of swelling studies are shown graphically in Double –Y plots showing dissolution profiles of zolpidem (ZP) release and swelling from matrices containing HPMC K4M and K100M grades combinations, (formulation codes C1,C2,C3, Fig. 1a. The percent uptake swelling or water uptake plots are shown in Fig. 1b. Similar plots are shown in Fig. 2a and Fig. 2b for formulation codes D1, D2, D3, containing HPMC K4M and K15M combinations with different ratios and Fig. 3a and Fig. 3b for formulation codes E1, E2, E3, containing HPMC K15M and K100M combinations with different

ratios.The dissolution parameters of varied formulation with different ratios of polymer combinations obtained during studies are shown in Table 3. In order to elucidate the release mechanism, the data were fitted to equation described by Peppas and Korsmeyer ($Mt/M \alpha Kt^n$). The value of release rate exponent (n) is a function of geometric shape of the drug delivery device. The results indicate that the mechanism of release is influenced greatly by the polymer concentration of the formulations as can be seen from the r^2 values and n was generally in accordance with these indications. The release is mainly determined by the Fickian diffusion which is also confirmed from the n values. Formulation C1 has n= 0.504, C2 has n=0.453 and C3 has n=0.444 indicating that the release mechanism is very close to Fickian transport i.e. belong to the Higuchi model.

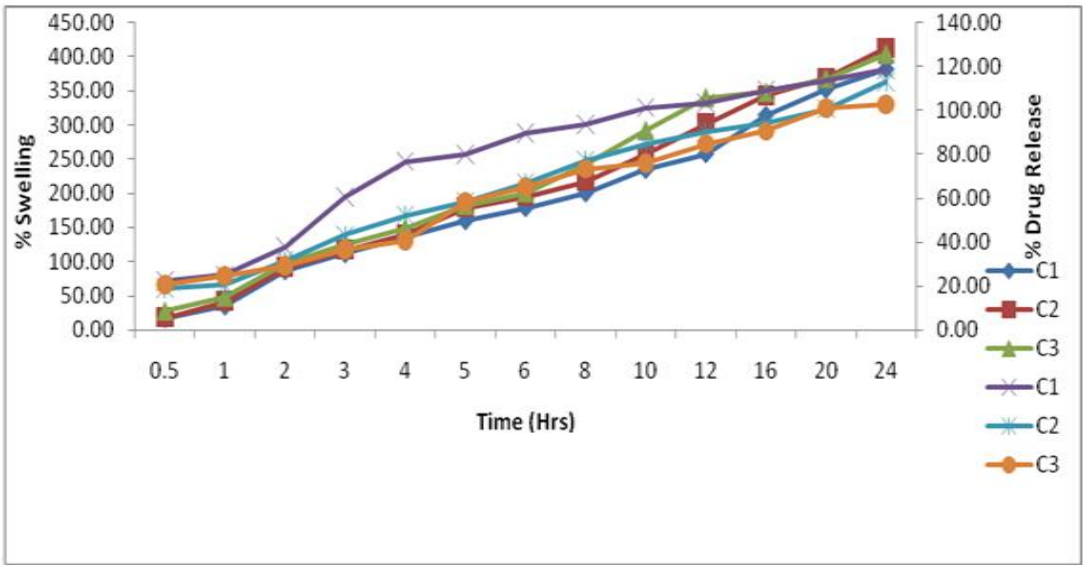

Fig. 1a. Double-Y Plots showing dissolution profiles of drug release and swelling for matrices containing HPMC K_4M and HPMC $K_{100}M$ (C_1-C_3) batches

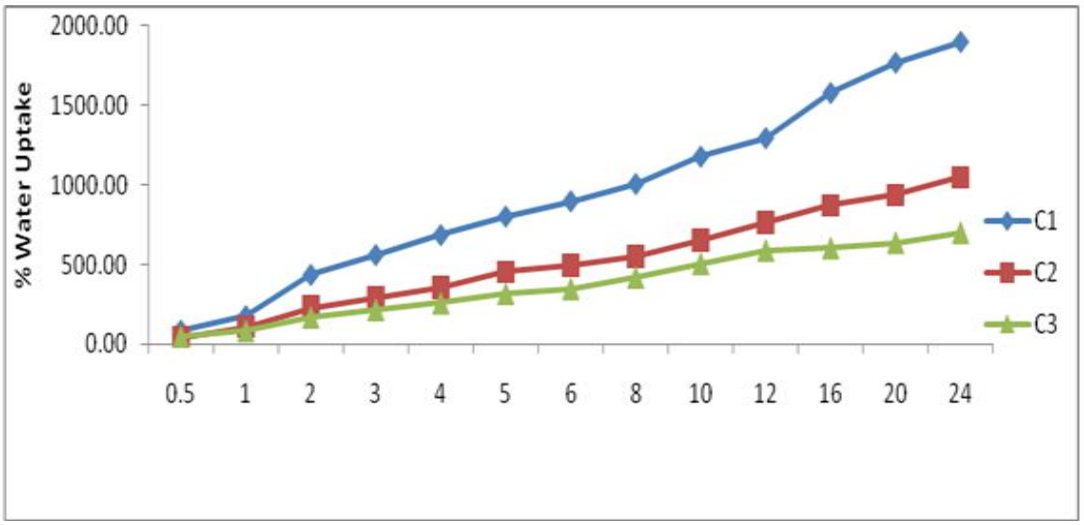

Fig. 1b. Plot of water uptake by HPMC K_4M and HPMC $K_{100}M$ matrices as a function of time

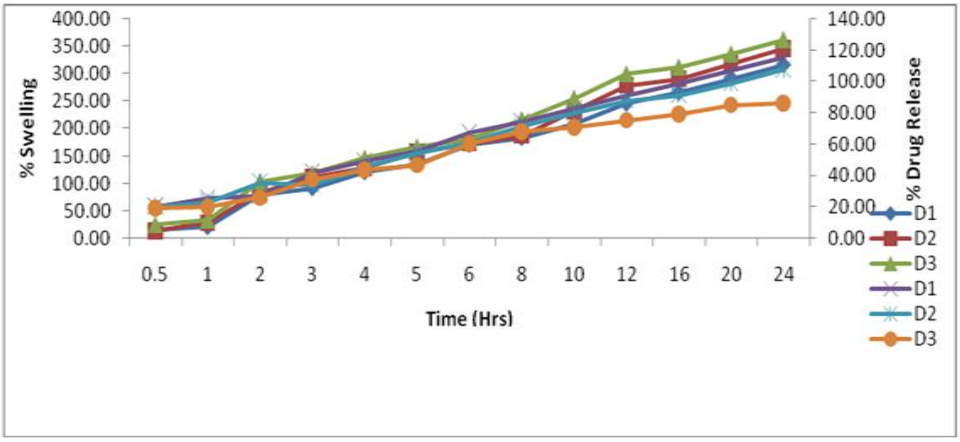

Fig. 2a. Double-Y Plots showing dissolution profiles of drug release and swelling for matrices containing HPMC K_4M and HPMC $K_{15}M$ (D_1-D_3) batches

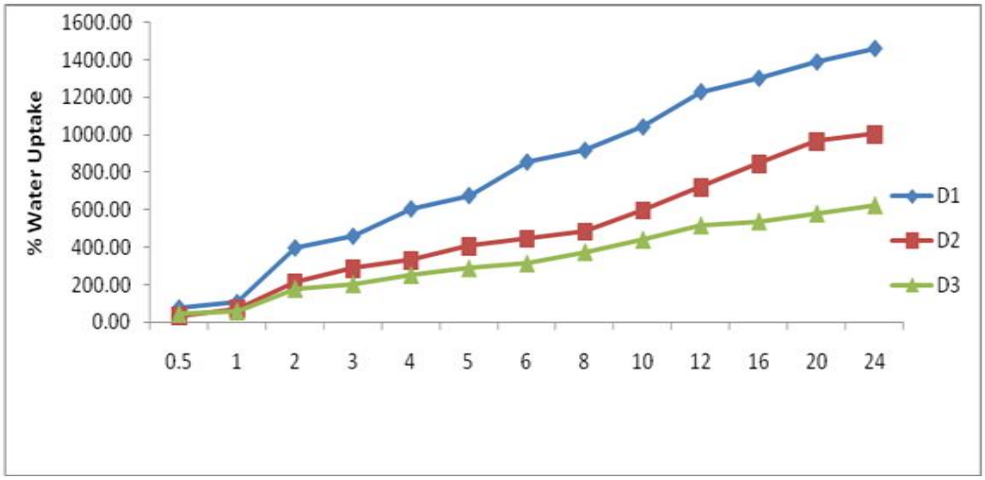

Fig. 2b. Plot of water uptake by HPMC K_4M and HPMC $K_{15}M$ matrices as a function of time

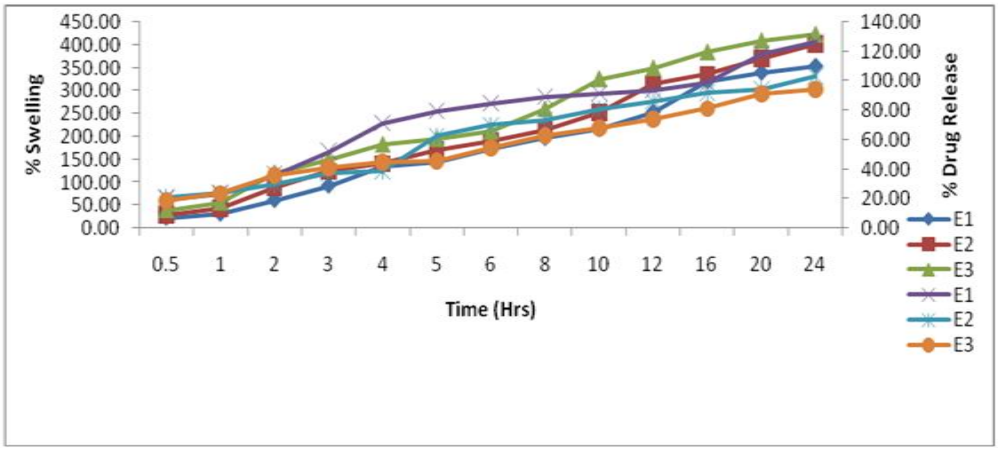

Fig. 3a. Double-Y Plots showing dissolution profiles of drug release and swelling for matrices containing HPMC $K_{15}M$ and HPMC $K_{100}M$ (E_1-E_3) batches

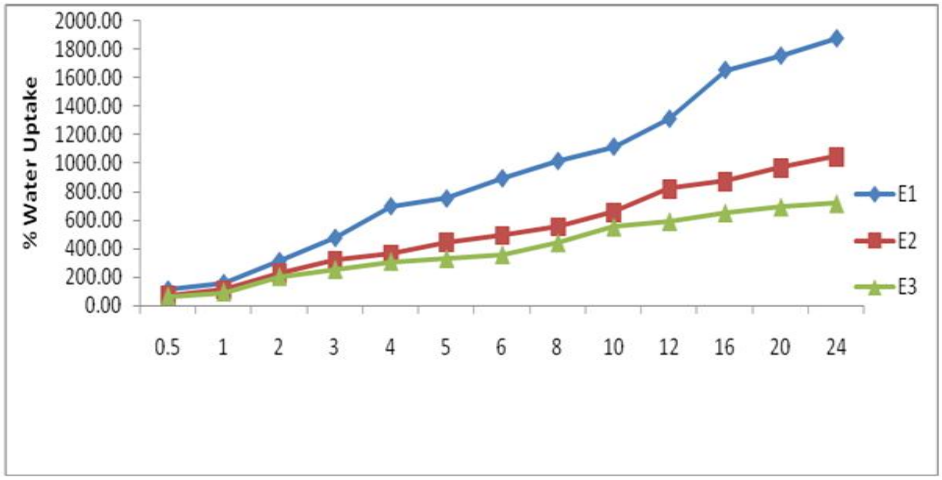

Fig. 3b. Plot of water uptake by HPMC $K_{15}M$ and HPMC $K_{100}M$ matrices as a function of time

Table 3. Dissolution parameters of varied formulations with different polymer ratio combinations

Formulation code	n	k	MDT	Rel 12 hr	Rel 24 hr	r^2	da/dt
C1	0.504	0.295	3.761	96.54	N.C.	0.986	1.247
C2	0.453	0.254	6.393	84.27	101.35	0.979	0.927
C3	0.444	0.235	8.009	75.00	84.82	0.977	0.817
D1	0.551	0.312	2.921	104.00	N.C.	0.974	1.449
D2	0.547	0.305	3.093	102.73	N.C.	0.975	1.382
D3	0.459	0.257	6.050	86.00	102.08	0.971	0.955
E1	0.508	0.288	3.885	93.05	N.C.	0.968	1.130
E2	0.444	0.235	8.008	74.99	84.82	0.977	0.817
E3	0.431	0.218	10.22	63.50	91.58	0.980	0.790

In this investigation it has been demonstrated that an inverse relationship exists between the drug release rate and matrix-swelling rate. When the amount of HPMC in the matrix is high, wetting improves and water uptake into matrices is enhanced. The higher amount of HPMC causes a greater degree of swelling. This in turn reduces the drug release, as the diffusional path length of drug is now longer. Conversely, reduction in the amount of HPMC reduces the degree of swelling and the thickness of gel layer. This enables faster drug release. Similar results are observed with the different viscosity grades of HPMC formulations, viz D1, D2, D3 and E1, E2, E3. HPMC of higher viscosity grades swells to greater extent and has greater intrinsic water uptake property than that of the lower viscosity grades.

4. CONCLUSION

Swelling studies reveals an inverse relationship between swelling and drug release. The rational combination of different grades of HPMC tends to provide quite regulated release of Zolpidem tartrate over an extended period of time. The diffusional exponent n generally agreed with the release kinetic employed and decreased with polymer concentration.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 7
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Evaluating the Characteristics of Blood Pressure Variability in Subjects with Chronic Kidney Disease Stage III in Diabetic or Non-diabetic Patients

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ABSTRACT

Aim: The aim of this study was to understand the relationship between circadian rhythm of blood pressure (BP) and renal function characteristics in subjects with chronic kidney disease (CKD) Stage III of diabetic and non-diabetic etiology.
Materials and Methods: A total of 30 CKD - hypertensive patients without diabetes and 30 Type 2 diabetic patients with overt diabetic nephropathy (DN) were enrolled in this study. The values of BP variability were obtained from 24 h ambulatory BP monitoring.
Results: As a result of a comprehensive examination of patients and statistical processing of data, it was found that in the group of patients with DN the level of albumin to creatinine in the urine was significantly higher than in the group of patients with non-DN (4.08 ± 6.15) mg/g and (1.43 ± 2.94) mg/g, respectively, significantly higher than triglycerides compared with the group of non-DN (2.72 ± 1.53) mmol/L and (1.55 ± 1.14) mmol/L, respectively. An interesting regularity was that patients with non-DN had a tendency to drop their BP in the morning, and thus the morning rise in systolic BP (SBP) and diastolic BP (DBP) in this group of patients was negative (−4.88 ± 21.35) mm Hg and (−70.88 ± 14.35) Hg, respectively. In patients with DN, these parameters exceed the norm and make up for SBP (66.02 ± 21.48) mm Hg and for DBP (57.13 ± 12.75) mm Hg. The mean daily diastolic pressure in the group of patients with DN was significantly higher than in the group of non-DN ([124.50 ± 33.78] and [111.50 ± 11.5] mm Hg, $P < 0.05$), the SBP variability was significantly lower than in the group with non-DN (13.67 ± 2.99) mm Hg and (16.35 ± 3.69) mm Hg, respectively.
Conclusions: In patients with DN with comparable values of glomerular filtration rate, higher albumin to creatinine ratio, lipid profile disorders were significantly more frequent than in the group of patients with the non-diabetic renal disease. The mean daily diastolic pressure in the group of patients with DN was significantly higher than in the group of non-DN ([124.50 ± 33.78] and [111.50 ± 11.5] mm Hg, $P < 0.05$), the SBP variability was significantly lower than in the group with non-DN (13.67 ± 2.99) mm Hg and (16.35 ± 3.69) mm Hg, respectively. DN patients have the more obvious disorders of triglycerides level in the lipid pattern than in the non-diabetic kidney damage group with the equal GRF values.

Keywords: 24 h ambulatory blood pressure; blood pressure variability; diabetic nephropathy; glycemic control; glycated hemoglobin; hypertension.

1. INTRODUCTION

The increase of blood pressure (BP) in diabetic of the 2nd type is associated, in most cases, with the presence of diabetic nephropathy (DN), and the basis of hypertension is damage to the renal

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parenchyma, accompanied by the formation of nodular or diffusive glomerulosclerosis [1]. 80% of patients with DN have a rise of BP even in the early stage of nephropathy, which is a significant cause of cardiovascular mortality in this group of patients [2]. Hypertension is a common problem in patients with CKD, and its incidence and prevalence increase with declining glomerular filtration rate (GFR) [3,4]. The reason of damage to the glomerulus of the patients with the 2nd type of diabetes is progressive microangiopathy, which leads to a disturbance of autoregulation of the intrarenal pressure by the kidney, and, as a result of this, the development of microalbuminuria and increased pressure in the glomerulus. The increase in arterial pressure of these patients is secondary compared with microalbuminuria and with increased intra-glomerular pressure, and it occurs under the influence of two mechanisms:

1. Increase in the amount of metabolic sodium and the volume of extracellular fluid.
2. Increased sensitivity of resistant vessels to noradrenaline and angiotensin II [1].

Circadian BP variation has been recognized for decades and has been documented in several studies using either invasive or noninvasive BP recorders in unrestricted or ambulant subjects [5-9]. According to the data of the diabetes control and complications trial research group (DCCT, 2004), the role of metabolic disorders, mainly hyperglycemia, in the pathogenesis of DN has been convincingly proved [2]. Such concept as “without diabetes, there are no diabetic complications” is the basis in understanding the nature of complications of diabetes, includes nephropathy. However, in a number of researches we can see that in the process of the development of DN, the direct dependence of the progression of nephropathy from the level of compensation of carbohydrate metabolism is lost [10]. It seems that the pathological process in the kidney gains its independent value [11]. it is known that progression of diabetic kidney disease correlates closely with level of hyperglycemia [12-14], and improving glycemic control decreases the rate of progression of diabetic kidney disease and loss of kidney function [15,16]. At the same time, a growing number of research results show the importance of dyslipidemia in the genesis of diabetic kidney damage already at the early stages of the development of diabetes. The system of accounting for standard risk factors is used when forming a risk group, whose role has been convincingly proven in different studies such as sex, age, body mass index, serum urine nitrogen, the values of the albumin/urine creatinine index, and BP indicators. However, there is still no explanation for the differences in the clinical features of kidney damage between a group of patients with diabetic and non-DN or differences between subgroups with different levels of glycated hemoglobin (HbA1c) [17]. The adoption of a screening program for DN in the framework of the Saint Vincent Declaration is a significant breakthrough in the diagnosis of the preclinical stage of nephropathy. According to this program, the main laboratory criterion for early DN stage is microalbuminuria, and according to the latest recommendations the albumin/ creatinine ratio in urine [18]. An urgent task in diabetology remains the evaluation of the functional state of the structures of the medullary substance of the kidney, and the determination of their concentration ability [19]. This is due to the fact that standard methods of assessing of kidney function, which is used in non DNs, are not informative in diabetes. It is determined that the basis for the prevention and treatment of DN is the achievement and maintenance of stable metabolic compensation of violations not only of carbohydrate but also of lipid metabolism [20]. Subsequent studies of the population as a whole [21] and hypertensive maintenance of stable metabolic compensation of violations not only of carbohydrate but also of lipid metabolism [8]. Subsequent studies of the population as a whole [21] and hypertensive cohorts [22] have generally confirmed, that increased night BP was an independent predictor of a high level of cardiovascular complications, especially in patients with DN [23,24]. It is considered that arterial pressure physiologically is regulated by various complex factors (e.g., environmental stimulation, genetic factors, autonomic nervous system, increased activity of the renin-angiotensin-aldosterone system, endothelial dysfunction, aging, prolonged smoking, excessive alcohol ingestion, obesity, caloric overload, emotions, and inflammation) [17,20,25]. However, the correlation between these factors and their effect on hypertension and BP variability is not studied sufficiently nowadays [26].

The indicants of BP variability were recognized as independent predictors of cardiovascular cases, which are independent of indicants of mean arterial pressure [26]. It was proved that the indices of the variability of arterial pressure depend on the activity of the sympathetic nervous system and changes in arterial extensibility [22]. Therefore, the hyperactivity of the sympathetic nervous system, the

violation of baroreflex sensitivity, and arterial stiffness inherent to diabetes mellitus - all these factors contribute to the increase in the variability of arterial BP (ABP), thereby aggravating the damage of target organs, [26] and increase the incidence and severity of cardiovascular cases [21].

However, nowadays the peculiarities of the ABP variability in patients with DN have been studied extremely little. In this research, we have analyzed 24-h BP monitoring (BPM) of patients with diabetic and non DNs to clarify the peculiarities of pressure profiles in these patient groups and identifying factors that can potentially affect the BP variability of patients with DN.

1.1 The Objective

The objective is, on the one hand, to estimate in a comparative aspect the relationship between the daily ABP rhythm, and, on the other hand, the renal function of patients with III Stage of diabetic and non-diabetic etiology.

1.2 Research Tasks

1. To study the peculiarities of the daily profile of arterial pressure of patients with III Stage of chronic kidney disease (CKD) of diabetic and non-diabetic etiology.
2. To determine the factors those most strongly affect the indicators of the daily profile of BP of patients with III Stage of CKD of diabetic and non-diabetic etiology.

2. MATERIALS AND METHODS OF RESEARCH

This study includes patients aged ≥18 years, with essential hypertension of 1–2°, diagnosed in a hospital based on criteria of hypertension according to the recommendations of the Ukrainian Cardiology Association (2012) and the clinical recommendations of the European Society of Hypertension (2013) [21] and CKD of diabetic and non-diabetic etiology of III Stage (according to the NKF K/DOQI classification, 2012, and the classification of the Ukrainian Association of Nephrologists [2013]) [27]. The functional state of the kidneys was assessed by the glomerular filtration rate according to the formula CKD-epidemiology, as well as the ratio of albumin to creatinine in urine. 60 patients were examined: 30 patients with DN and 30 CKD patients with non-diabetic etiology (24 patients with chronic glomerulonephritis and 6 patients with chronic pyelonephritis). The median age of patients was approximately 48.6 ± 5.1 years. The 24-h BPM was carried out on the apparatus BAT41-2 with an oscillometric method for 24 h with a measurement interval of 15/30 min day/night. All patients were instructed about the need to fill individual diaries, to record the time of falling asleep, the morning rise and other activities. Hence, the values of “daily” and “night” indicators of BP and pulse of patients were recorded in accordance with the time of wakefulness and sleep noted in their diaries. If patients had more than 20% of the errors in the measurement of BP or the absence of measurement values of ABP for more than 2 h in a row, then they should have daily BPM over the next 24 h. Values of systolic BP (SBP) >240 or <70 mm Hg and diastolic BP (DBP) >150 or <40 mm Hg. st. were removed from the profile as technical artifacts.

Exclusion criteria were: Patients who undergo dialysis or patients after kidney transplantation, patients with clinically significant heart disease (heart failure III-IV Stage according to NYHA, GB III, and myocardial infarction), stroke, stenosis of the renal arteries, hepatic dysfunction, pheochromocytoma, thyrotoxicosis, and hyperaldosteronism.

All patients with DN received standard therapy in the form of a combination of metformin and glimepiride/glibenclamide, while dieting and the scheme of physical activity.

According to the recommendations of the Ukrainian Association of Cardiologists Treatment of arterial hypertension included α- and β-blockers, angiotensin-converting enzyme or sartans, calcium channel blockers, and diuretics. Clinical examination of patients included collection of complaints, anamnesis, objective examination data, and biochemical and instrumental survey methods.

According to the level of HbA1c, patients with DN were divided into two subgroups: 1 Group (HbA1c <7%) and 2 Group (HbA1c ≥7%). The data, which characterized the clinical and laboratory indicators of patients with CKD III Stage of diabetic and non-diabetic etiology are presented in Table 1.

Table 1. Clinico-laboratorial findings in patients with III Stage CKD of diabetic and non-diabetic etiology

Index	Diabetic nephropathy	Non-diabetic nephropathy
Number of patients	30	30
Age	61.58±6.14	58.68±12.75
Sex (men/women)	38/22	30/21
Smokers	22 (36.8%)	16 (31.4)
Duration of diabetes (months)	114	-
Height-and-weight index (kg/m2)	28.24±2.15	23.44±3.23
Hemoglobin (g/l)	105.92±11.16	107.02±27.84
Hematocrit	0.43±0.04	0.42±0.08
Fasting glucose (mmol/l)	11.44±1.73	5.01±0.6*
HbA1c (%)	8.76±0.78	5.55±1.45*
BUN (mmol/l)	4.97±0.77	5.51±1.05
Creatinine (mmol/l)	198.75±12.08	237.30±14.05
Albumins/creatinine of urine	4.08±6.15	1.43±2.94*
GRF (ml/[min*1.73 m3])	31.96±3.55	22.53±3.05
Total cholesterol (mmol/l)	4.485±2.09	4.47±1.17
Triglycerides (mmol/l)	2.72±1.53	1.55±1.14*
Calcium (mmol/l)	2.45±0.11	2.09±0.13
Phosphorus (mmol/l)	1.13±0.17	1.23±0.54
SBP 24 h., mm Hg	140.83±10.83	139.12±18.90
Maximum day DBP, mm Hg	124.50±33.78	111.5000±11.5*
RMR in SBP, mm Hg	66.02±21.48	−54.88±21.35*
RMR in DBP, mm Hg	57.13±12.75	−70.8750±14.35*
SMR in SBP, mm Hg	54.83±2.48	24.8±2.36*
SMR in DBP, mm Hg	10.83±2.69	29.61±8.24*
Variability of SBP per 24 h, mm Hg	12.23±3.66	10.74±5.26*
Variability of DBP per 24 h, mm Hg	8.02±1.93	8.30±2.40
Day variability of SBP, mm Hg	20.71±6.34	20.5±5.26
Day variability of DBP, mm Hg	15.81±2.69	18.37±2.4
Night variability of SBP, mm Hg	13.67±2.99	16.35±3.69*
Night variability of DBP, mm Hg	12.25±2.41	14.38±3.09
Number of non-dipper patients	25 (83%)	21 (70%)*
Number of night-peaker patients	3 (10%)	0*

**Possibility of deviation in indexes comparing to the group of diabetic nephropathy patients (P < 0.05). HbA1c: Glycated hemoglobin, BUN: Blood urea nitrogen, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, RMR: Rate of morning rise, CKD: Chronic kidney disease, SMR: Speed of morning rise*

Statistical processing of data was carried out with the help of the software package Statistica 6.0 for Windows. The values of the indicators were: Median (Me), 25% - was the lowest quartile and 75% - was the upper quartile (IU [25% and 75%]).

Statistical differences were determined at a significance level of $P < 0.05$. The Mann–Whitney U-test was used for unrelated samples, and the Wilcoxon criterion for linked samples was used to compare the indices in this two groups.

3. RESULTS AND DISCUSSION

In the results of the study made in the group of DN patients, there was found the certain majority of patients with “non-dipper” (25 patients) and “night-peaker” (3 patients) day rhythms comparing to the

group of non-DN patients (21 and 0 correspondently). In the same group, the level of albumins in relation to creatinine in urine was accurately 185.3% higher than in the group of non-DN patients. Furthermore, in the group of DN patients there was found the accurate increase of triglycerides level comparing to the group of non-DN (2.72 ± 1.53 mmol/l or 1.55 ± 1.14 mmol/l correspondently). Estimating the indexes of daily BPM there draws attention the fact, that accurate differences first of all related to exponents of diastolic pressure - in such a way the level of average daily DBP in the DN patients group was certainly 11.7% higher than in the non-DN group. There was an interesting dependency that in patients with a non-DN there was found the tendency to the drop of BP in the morning instead of its rise as it should be within the norm, so the rate of SBP and DBP morning rise in this patient's group was negative. However, for the DN patients these exponents are above the norm and make (66.02 ± 21.48) mm Hg for SBP and (57.13 ± 12.75) mm Hg for DBP; nevertheless, this exponent will not be informative enough for the patients with a monotonous daily BP profile. Joint studying of the rate of morning rise and speed of morning rise (SMR) of BP gives the more full characteristics. As the SMR is an integral exponent and depends only on the rate and time of BP increase, it is not influenced by neither daily rhythm nor absolute values of BP which are not always maximum in the morning. Exponent of the speed of morning increase of SBP and DBP in diabetic and non-DN patients had the significant differences, exponents for SBP were accurately 121.1% higher in the DN group, and 173.4% more for DBP in the non-DN group. This phenomenon has the great clinical meaning, as in the period from 6:00 to 12:00 there is noted a leap of BP, increase of vascular tone which coincide with neurohumoral changes. At this time, there is also noted the only period during the day when there is defined the increase of thrombocyte aggregation, hypercoagulability, and reduction of fibrinolytic activity. In the morning, there is noted the physiological activation of sympathoadrenal and renin-angiotensin-aldosterone systems, increase of sympathetic and a decrease of parasympathetic activity. In such a way, the significant increase of BP in the morning in conjunction with neurohumoral changes may be the trigger of the range of well-known processes non-favorable regarding the cardiovascular complications. In such a way, the high rate and speed of BP increase in the early morning time are the independent risk factor for the left ventricular myocardial hypertrophy.

Besides, in the DN patients group, the exponents of night variability of SBP were accurately 19.6% lower than in the non-DN patient's group.

4. CONCLUSIONS

In the DN patients, there was found the tendency to increase of DBP. It is shown that alongside with the comparable reduction of the kidneys operant behavior the DN hypertension differs from the DN hypertension for the higher exponents of pressure load, frequency, and severity of disorder of daily BP rhythm with the lower values of variability and higher rate and speed of morning BP rise.

With the equal exponents of GRF is glomerulus filtration rate in DN patients, the level of urinary albumins/creatinine is significantly higher than in the non-diabetic kidney damage patients group.

DN patients have the more obvious disorders of triglycerides level in the lipid pattern than in the non-diabetic kidney damage group with the equal GRF values.

5. PROSPECTIVE OF THE FURTHER RESEARCH

There is expected to analyze the influence of the DPP-IV (sitagliptin) drug on the kidneys and liver function, lipid panel, and albumins/creatinine correlation of urine in patients with CKD of the III Stage of diabetic etiology.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 8
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Investigation on the Anti-ulcerogenic Effects and Anti-oxidative Properties of *Ceiba pentandra* Leaves on Alloxan-induced Diabetic Rats

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ABSTRACT

Several natural products, mostly of plant origin have been shown to possess promising activities that could assist in the prevention and/or amelioration of diseases. Gastric ulcer is an ulceration of the gastrointestinal tract which results from persistent erosions and damage of the stomach wall that might become perforated and develop into peritonitis and massive haemorrhage.
Aims: The current study was aimed to evaluate the protective effects of methanol extract of *Ceiba pentandra* leaves on indomethacin and ethanol induced gastric ulcer and on oxidative stress indices of alloxan-induced diabetic rats.
Study Design: Extraction and administration of graded doses of the extract
Place and Duration of Study: Department of Biochemistry, University of Nigeria, Nsukka. Enugu State, Nigeria, between May, 2011 and October, 2011.
Methodology: Extraction of *Ceiba pentandra* leaves was done using methanol. Twenty adult rats divided into five groups of four rats each were used for each of the ulcer studies. Gastric ulceration was induced in the rats by oral administration of indomethacin (50 mg/kg) and 95% ethanol (0.5 ml) thirty minutes after extract treatment, and the animals sacrificed 8 h later. For the diabetes study, thirty (30) albino rats divided into six (6) groups of five (5) rats each were used. Diabetes was induced by i.p injection of alloxan monohydrate (150 mg/kg) in overnight-fasted animals and the animals treated with varied doses (100, 200 and 400 mg/kg) of the extract for two weeks. Serum obtained from the diabetic rats was used for the determination of lipid profile and liver marker enzymes.
Results: Significant and dose dependent ulcer inhibition (70, 82 and 84%; 19, 53, and 58% for 100, 200 and 400 mg/kg of the extract respectively) was produced in all the extract-treated groups for the ulcer models used. There were significant decreases ($p<0.05$) in fasting blood glucose levels, liver marker enzymes, total cholesterol, low density lipoprotein and triacylglycerides in the serum of extract-treated groups compared with that of the diabetes-untreated group.
Conclusion: The findings in this study show that methanol extract of *Ceiba pentandra* leaves possesses potent anti-ulcerogenic and anti-oxidative properties and has potential for use as an herbal remedy for the treatment of gastro-intestinal ulcer and management of diabetes.

Keywords: Ceiba pentandra; diabetes; liver enzymes; lipid profile; gastric ulcer; indomethacin.

1. INTRODUCTION

Plants have not only provided mankind with food, clothing, flavours and fragrances, but have also been an indispensable source of natural products for relief and treatment of different ailments [1]. Plant-based systems continue to play an essential role in healthcare, and their use by different cultures has been extensively documented [2,3,4]. Several natural products, mostly of plant origin have been shown to possess promising activities that could assist in the prevention and/or amelioration of diseases. Gastric ulcer is an ulceration of the gastrointestinal tract which results from persistent erosions and damage of the stomach wall that might become perforated and develop into

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peritonitis and massive haemorrhage [5,6]. This occurs as a result of imbalance between some endogenous aggressive factors such as hydrochloric acid, pepsin, reactive oxygen species (ROS) and cyto protective factors, which include the mucus bicarbonate barrier, surface active phospholipids, prostaglandins (PGs), mucosal blood flow, non- enzymatic and enzymatic antioxidants [7-9]. The success of commercially available antiulcer drugs such as H_2- receptor antagonists and proton-pump inhibitors in the treatment of gastric ulcer is usually overshadowed by various side effects. This has led to the search for new anti-ulcerogenic agents from plants with low toxicity and minimal side effects. Diabetes mellitus (DM) is a metabolic disorder of multiple aetiology; characterized by chronic hyperglycemia with alterations in carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both [10-12]. Diabetes mellitus is a combination of heterogeneous disorders commonly presenting with episodes of hyperglycaemia and glucose intolerance, as a result of lack of insulin , defective insulin action, or both [13,14]. The major types of diabetes are Insulin-Dependent Diabetes Mellitus (IDDM) or type 1 diabetes, Non- Insulin Dependent Diabetes Mellitus (NIDDM) or type 2 diabetes and gestational diabetes [15]. The underlining causes of diabetic complications have been attributed to hyperglycemia which results in oxidative stress, alterations in enzyme activities, protein glycosylation and several structural changes [16-17]. The pathogenesis of diabetes mellitus and the possibility of its management by existing therapeutic agents without any side effects is still a challenge for the medical system and have stimulated great interests in recent years. This has led to increasing demand for natural products with anti-diabetic activity with fewer side effects. *Ceiba pentandra* (L) Gaertner, known as silk cotton tree belongs to the Bombacaceae family [18]. The plant is widely spread around the world and is reputed in the African traditional medicine [19]. Previous studies on various morphological parts of the plant have shown that the plant possesses hypoglycemic effects [20], anti ulcerogenic effects [21-22] and anti-diarrhoea properties [23]. The plant is also used as a diuretic and effective remedy against headache, dizziness, constipation and rheumatism [24-25]. This present study was undertaken to evaluate the protective effect of methanol extract of *Ceiba pentandra* leaves on indomethacin and ethanol induced ulcers and on oxidative stress indices of alloxan-induced diabetic rats.

2. MATERIALS AND METHODS

2.1 Materials

Fresh leaves of *Ceiba pentandra* were collected in the month of May, 2011 from the botanical garden of University of Nigeria, Nsukka. The leaves were identified and authenticated by Mr. Alfred O. Ozioko, a Botanist at the International Centre for Ethnomedicine and Drug Discovery, (InterCEED) Nsukka. A voucher specimen was deposited in the herbarium unit of the Department of Botany, University of Nigeria, Nsukka. The leaves were sun-dried for one week and milled to a coarse powder using a milling machine. The powdered leaves (1100 g) was macerated in methanol (5000 ml) for 48 h and filtered with Whatmann No. 1 filter paper. The filtrate obtained was dried in a rotary evaporator (IKA, Germany) at an optimum temperature of 50°C and the dried crude extract of weight 52.6 g that was obtained was stored in a sterile container until use.

2.2 Phytochemical and Proximate Analyses *Ceiba pentandra* Leaves

Qualitative phytochemical analyses of *Ceiba pentandra* leaves were carried out using the method of Harborne [26] and Trease and Evans [27]. The proximate analysis for moisture, ash and carbohydrate contents were determined as described by AOAC [28]. All determinations were done in triplicates and the results were expressed as averages of percent values on dry weight basis.

2.3 Animals

Swiss albino mice (22±10 g) of both sexes and adult male albino rats (150±10 g) obtained from the animal house of the Faculty of Biological Sciences, University of Nigeria, Nsukka were used for the studies. The animals were acclimatized for 7 days under standard environmental conditions, with a 12 h light/dark cycle and maintained on a regular feed (Vital feeds, Nigeria) and water *ad libitum*. The research was conducted in accordance with the ethical rules and recommendations of the University

of Nigeria committee on the care and use of laboratory animals and the revised National Institute of Health Guide for Care and Use of Laboratory Animal (Pub No.85-23, revised 1985).

2.4 Acute Toxicity Study

The acute toxicity test of the plant extract was carried out by the method of Lorke [29]. Eighteen Swiss albino mice starved of food for 18 h but allowed access to water were used for the study. They were divided into six groups of three mice each and administered intraperitoneally (i.p.) with the plant extract at varied dose levels (10, 100, 1000, 1600, 2900 and 5000 mg/kg). The animals were then observed for nervousness, dullness, in-coordination and or mortality for 24 h.

2.5 Anti-ulcer Activity

Two experimental models of inducing gastric ulcer were used to assess the ulcer preventive properties of *Ceiba pentandra* leaves. Twenty adult rats randomly divided into five groups of four rats each were used for each model. They were deprived of food for 18 h and treated orally with normal saline and varying doses of methanol extract of *Ceiba pentandra* leaves. Group 1 (normal control) was administered orally with normal saline (5 ml/kg). Groups 2, 3 and 4 received the plant extract at varied dose levels: 100, 200 and 400 mg/kg respectively while group 5 (reference group) received the standard drug treatment of 100 mg/kg of ranitidine (Zantac®).

2.5.1 Effect of *Ceiba pentandra* leaves on indomethacin-induced ulcer

This assay was carried out using the method of Ubaka et al. [30]. The animals were deprived of food for 18 h and treated orally with normal saline and varying doses of the plant extract as stated above. Thirty minutes later, 50 mg/kg of indomethacin was administered (p.o) to the rats. After 8 h, each animal in the groups was sacrificed by chloroform anaesthesia and the stomach removed and opened along the greater curvature, rinsed with normal saline and pinned flat on a board. Erosions formed on the glandular portions of the stomach were viewed with a hand lens (x10), counted and the ulcer scored using the following scale:

No ulcer	= 0 point
Superficial ulcer 1-2 mm in length	= 1 point
Elongated erosions 3-4 mm in length	= 2 points
Deep ulcer and perforations	= 3 points

The sum of all the lesions/ulcers in all the animals for each group (total ulcer score) was divided by 10 to obtain the mean ulcer index. The percent ulcer inhibition was calculated relative to control thus:

$$\% \text{ ulcer inhibition (\% U.I)} = \left(1 - {Ut}/{Uc}\right) \times 100$$

Where Ut and Uc represents the ulcer index of the treated and control groups respectively.

2.5.2 Effect of *Ceiba pentandra* leaves on ethanol–induced gastric ulcer

Ulcer was induced according to the method of Shokunbi and Odetola, [31]. Twenty rats were randomly divided into 5 groups of 4 rats each and pre-treated orally with the extract as stated above before ulcer induction. Ulcer lesion was established with 0.5 ml of 95% ethanol (p.o.). After 4 h, the animals were killed by cervical dislocation. The stomachs were removed and opened along the greater curvature and macroscopic examination carried out with a hand lens (x10).

2.6 Induction of Diabetes

Thirty (30) albino rats divided into six (6) groups of five (5) rats each were used for the study. Diabetes was induced by i.p injection of alloxan monohydrate (Sigma St. Louis, USA) (150 mg/kg) in overnight-fasted animals. Diabetes was confirmed seven (7) days later in the alloxan-induced animals showing

Fasting Blood Glucose, (FBG) level≥200 mg/dl (11.1mmol/L) and was monitored on blood obtained from tail vein puncture using an automated glucose sensor machine – Glucometer (AccuChek Active).

2.6.1 Treatment of animals

Rats in group 1 (normal control) were administered normal saline for 2 wks. Groups 3, 4 and 5 were diabetes- induced rats treated p.o. with 100, 200 and 400 mg/kg of the extract respectively while groups 2 (diabetes -untreated control) and 6 (standard drug control) were diabetes-induced rats administered with normal saline (5 ml/kg) and 5 mg/kg of glibenclamide (standard anti-diabetic drug) respectively for 2 wks. Blood samples (20 µl) were obtained from the tail tip of fasted rats and the blood glucose level determined using a glucometer (AccuChek Active).

2.6.2 Preparation of serum

At the end of the experiment, blood was collected from the rats by carotid bleeding into centrifuge tubes. The blood samples were centrifuged and the clear serum supernatant was used freshly for the assessment of some biochemical and liver function tests.

2.6.3 Assessment of liver marker enzymes and other biochemical parameters

The activities of alanine amino transferase (ALT) and aspartate amino transferase (AST) were estimated by the Reitman-Frankel colorimetric method [32] using Quimica Clinica Applicada QCA test kits. Alkaline phosphatase (ALP) was measured by the method of Klein et al. [33] (Serum cholesterol and triacylglycerides were determined by the method of Allain et al. [34] and low density lipoprotein (LDL) was by the method of Assmann et al. [35].

2.7 Statistical Analysis of Data

Data obtained were analyzed by one-way ANOVA using SPSS version 17.0 (SPSS Inc. Chicago, IL. USA). All values were expressed as mean±SEM. Differences between means were assessed by Duncan's Multiple Range Test (DMRT). Differences were considered statistically significant when $P \leq 0.05$.

3. RESULTS

3.1 Acute Toxicity Test of the Extract

Result of acute toxicity test of the extract show no mortality in all the groups of mice administered with methanol extract of *C. pentandra* leaves up to the dose of 5000 mg/kg body weight Table 1.

Table 1. Acute toxicity test of the extract

Group	No of mice used	Dose (mg/kg)	Dead (%)
1	3	10	0
2	3	100	0
3	3	1000	0
4	3	1600	0
5	3	2900	0
6	3	5000	0

3.2 Phytochemical Analysis of the Methanol Extract of *Ceiba pentandra* Leaves

Phytochemical analysis of the methanol extract of *C. pentandra* leaves showed the presence of bioactive compounds such as flavonoids, tannins glycosides, phenols, alkaloids, saponnins, steroids and terpernoids Table 2.

Table 2. Phytochemical constituents of methanol extract of *Ceiba pentandra* leaves

Constituents	Bioavailability
Alkaloid	++
Flavonoid	+++
Glycosides	+++
Saponnins	++
Tannins	+++
Resins	-
Steroids	++
Terpenoids	++
Acidic compounds	_
Phenols	++

++++ - Abundantly Present
+++ - Present in very high concentration
++ - Present in moderately high concentration
+ - Present in small concentration
- - Absent

3.3 Proximate Analysis of *Ceiba pentandra* Leaves

Proximate analysis of *Ceiba pentandra* leaves for moisture, ash and carbohydrate showed 53, 2.5 and 24% respectively while that of fats and protein showed 12 and 8.5% respectively Table 3.

Table 3. Proximate analysis of *Ceiba pentandra* leaves

Constituents		Quantity (%)
Ash		2.5 ± 0.08
Moisture	53 ± 0.16	
Fats	12 ± 0.10	
Protein	8.5 ± 0.05	
Carbohydrate	24 ± 0.08	

3.4 Indomethac in Induced Ulcer

Indomethacin produced ulcers in all the rats of the groups. Ulcers produced in this model were seen as large black sores (Fig. 1a). Potent and dose dependent ulcer inhibition was observed in all the groups treated with *Ceiba pentandra* leaf extract (Fig. 1b). This was also evidenced by the significantly ($P \leq 0.01$) reduced ulcer indices of the groups, which at 200 (0.45±0.02) and 400 mg/kg (0.40±0.01) were comparable to that obtained for ranitidine (0.42 ±0.20), the standard anti- ulcer drug used. The percent ulcer inhibitions exerted by the extracts were 70, 82 and 84% for 100, 200 and 400mg/kg of the extract respectively Table 4.

Fig. 1a. Black ulcer sores induced by indomethacin (control group)

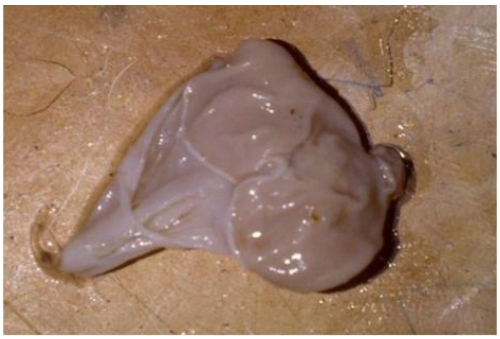

Fig. 1b. Reduced ulcer sores (400mg/kg extract)

Table 4. Effect of Ceiba pentandra leaves on indomethacin-induced ulcer in rats

Treatment	Dose (mg/kg)	No of rats	Mean Ulcer index	% ulcer inhibition
Normal saline	5 ml/kg	4	2.50±0.52	-
Extract	100	4	0.75± 0.02*	70
	200	4	0.45± 0.02*	82
Ranitidine	400	4	0.40± 0.01*	84
	100	4	0.42± 0.20*	83

*Values shown are mean ± SEM (n = 4). Level of significance * = P < 0.05*

3.5 Ethanol-induced Ulcer

Ethanol produced gastric ulceration in all the animals in the groups. Ulcers produced in this model were seen as reddish sores on the gastric epithelial walls (Fig. 2a). Treatment with the extract reduced ulcer formation in the gastric mucosa of the rats (Fig. 2b). There was significant ($P \leq 0.05$) reduction in the ulcer index of the groups treated with 200 (1.33±0.057) and 400 mg/kg (1.20±0.087) of the extract when compared with that of the control group (2.86±0.430). The percent (%) ulcer inhibitions exerted by the extracts were 19, 53, and 58% for the 100, 200 and 400 mg/kg respectively Table 5.

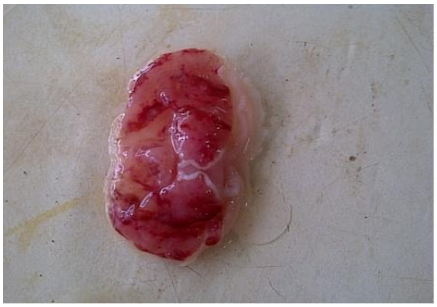

Fig. 2a. Reddish ulcer sores on the gastric walls induced by aspirin (Control group)

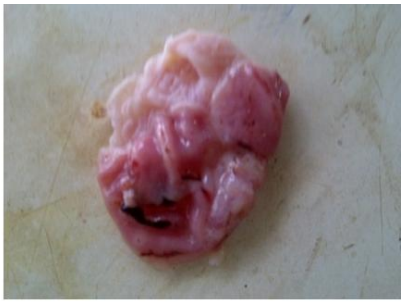

Fig. 2b. Reduced ulcerative sores on the gastric walls of rats (100 mg/kg extract)

Table 5. Effect of *Ceiba pentandra* stem bark extract on ethanol-induced ulcer in rats

Treatment	Dose (mg/kg)	No of rats	Mean Ulcer index	% ulcer inhibition
Normal saline	5 ml/kg	4	2.86± 0.430	-
Extract	100	4	2.33± 0.386	19
	200	4	1.33± 0.057*	53
Ranitidine	400	4	1.20± 0.087*	58
	100	4	1.23± 0.082*	57

*Values shown are mean ± SEM (n = 4). Level of significance *= P < 0.05*

3.6 Effect of *Ceiba pentandra* Leaves on Blood Glucose and Oxidative Stress Markers in Alloxan-induced Diabetic Rats

Data in Table 6 show that the diabetes-induced rats had significantly increased ($P<0.01$) levels of blood glucose before extract administration when compared to that of the non-diabetic (normal) control rats. Treatment of the animals with methanol extract of *Ceiba pentandra* leaves produced significant dose-dependent decreases ($P<0.05$) in the glucose level when compared with the diabetes-untreated rats. The reduced glucose levels obtained with extract treatment was comparable to that obtained with the treatment with glibenclamide. Result in Table 7 show significantly increased

(*P*<0.01) activities of the liver enzymes (ALP, ALT, AST) in the diabetes -untreated rats when compared to that of the non-diabetic (normal) control rats. Treatment of the animals with 200 and 400 mg/kg of the methanol extract of *Ceiba pentandra* leaves produced significant dose-dependent decreases (*P*<0.05) in the liver enzymes with values comparable to those of glibenclamide-treated and normal control groups. Serum levels of cholesterol, TAG and LDL were significantly increased (*P*<0.05) while HDL significantly decreased (*P*<0.01) in the diabetes-untreated rats when compared to the normal rats. Treatment with the extract significantly reduced (P<0.05) the cholesterol, TAG and LDL levels and significantly increased (*P*<0.05) HDL of all the extract -treated groups with values comparable to those of glibenclamide treated and normal control groups.

Table 6. Blood glucose of alloxan induced-diabetic rats before and after treatment with methanol extract of *Ceiba pentandra* leaves and glibenclamide

Treatment groups Parameters	Normal control	Diabetes-untreated	Diabetes + 100 mg/kg	Diabetes + 200 mg/kg	Diabetes + 400 mg/kg	Diabetes + glibenclamide
Glucose conc. (mg/dl) before extract administration	134± 8.58^{a}	168 ±8.02^{b}	169 ± 10.57^{b}	170 ± 6.87^{b}	167 ± 12.32^{b}	171 ± 9.89^{b}
Extract administration days extract administration	132 ± 12.24^{a}	166 ± 9.54^{b}	136 ± 8.51^{a}	133 ± 10.05^{a}	126 ± 6.68^{a}	129 ± 8.68^{a}

Means with different lower case superscripts (a, b, c) across the row i.e. between groups are significantly different at P<0.05

Table 7. Effect of methanol extract of *Ceiba pentandra* leaves on oxidative stress markers in alloxan induced-diabetic rats

Treatment groups Parameters	Normal control	Diabetes untreated	Diabetes + 100 mg/kg	Diabetes + 200 mg/kg	Diabetes + 400 mg/kg	Diabetes + glibenclamide
Alanine amino transferase (U/L)	33 ± 4.3^{a}	78 ± 2.83^{c}	81 ± 7.07^{c}	45.5 ± 4.95^{a,b}	45.5 ± 1.71 ^{a}	55.5 ± 1.96^{b}
Aspartate amino transferase (U/L)	52.5 ± 9.19^{a}	71 ± 5.66^{b}	54 ± 2.12^{a}	45 ± 3.4^{a}	44.5 ± 6.36 ^{a}	46 ± 4.24^{a}
Total cholesterol (mg/dl)	24.5 ± 6.4^{a}	62.5 ± 7.8 ^{b}	34 ± 1.4^{a}	30.5 ± 6.4^{a}	29 ± 8.5 ^{a}	31.5 ± 7.8^{a}
Triacylglyceride (mg/dl)	7.5 ± 2.1^{a}	40 ± 7.1^{c}	22 ± 2.8^{b}	17 ± 0.7^{a,b}	13.5 ± 0.7 $^{a, b}$	25.5 ± 2.1^{b}
Low Density Lipoprotein (mg/dl)	19.5 ± 4.9^{a}	37 ± 1.4^{b}	22 ± 0.3^{a}	19 ± 4. ^{a}	21 ± 0.7 ^{a}	25.5 ± 4.9^{a}
High Density Lipoprotein (mg/dl)	40.0 ± 1.4^{a}	2.5 ± 0.3^{c}	7.5 ± 2.8^{c}	15 ± 5.6 ^{b}	33 ± 0.7 ^{a}	16.5 ± 1.4^{b}

Means with different lower case superscripts ($^{a, b, c}$) across the row i.e. between groups are significantly different at P<0.05

4. DISCUSSION

Natural products derived from plant sources have always been the main sources of new drugs for the treatment of various diseases [36]. *Ceiba pentandra*, a multi- purpose and herbaceous plant native to Mexico, Central America and tropical West Africa has been reported to have diverse medicinal and pharmacological uses. In this work, an attempt was made to determine the effect of the methanol extract of *Ceiba pentandra* leaves on indomethacin and ethanol-induced ulcers and on oxidative stress markers of alloxan-induced diabetic rats. Result of the acute toxicity study showed that the plant leaves were non-toxic and relatively safe to the experimental animals. Pretreatment of rats with methanol extracts of *Ceiba pentandra* leaves before induction with indomethacin/ethanol significantly

decreased the ulceration in the gastric linings of the animals when compared to the control group. Indomethacin induces ulcer in rats either by direct mucosal injury which involves the breaking of the mucosal barrier and exposure of the underlying tissue to the corrosive action of acid and pepsin or by a decrease in endogenous gastric prostaglandin production and release through COX-1 and COX- 2 inhibition [6,37]. The naturally occurring prostaglandins are important for the production of gastric bicarbonate and mucous which are the key components of the stomach protective barrier [8]. They inhibit acid secretion, maintain gastric microcirculation and increase mucosal blood flow. Thus, continuous generation of prostaglandins by the mucosa is crucial for the maintenance of mucosal integrity and protection against ulcerogenic and necrotizing agents and its inhibition triggers damage to the mucosal lining [6,38]. Pre-treatment with *Ceiba pentandra* extract in this study, reduced the indomethacin-induced ulcer in the rats perhaps by the mechanism of increased endogenous prostaglandin synthesis which in turn promoted mucus secretion and enhanced the mucosal barrier against the action of the ulcerogenic agent. Ethanol administration to experimental rats damages the gastrointestinal mucosa by micro- vascular injury leading to increased vascular permeability, gastric mucus depletion and edema formation. It is metabolized in the body to release superoxide anion and hydroperoxyl free radicals which are involved in the mechanism of acute and chronic ulceration of the gastric mucosa [39,40]. The inhibition of the ethanol-induced ulcer by the *Ceiba pentandra* leaves could be due to the plant's antioxidant property and ability to chelate free radicals and reactive oxygen species, thus reducing oxidative damage to the mucosal membrane and epithelial cells. This activity could be linked to the presence of some antioxidant constituents; flavonoids, saponnins and tannins, in the plant. These substances characterized by their polyphenolic nature, have been reported to have cytoprotective and antiulcer activities in other plants [41,42]. Ulcer preventive effect of the plant could also be attributed to the presence of alkaloids in the plant leaves. Reports have shown that plant derived alkaloids have significant activity in acute and chronic gastric ulcers in rats. These alkaloids increase free mucus and prostaglandin production and show a reduction in hemorrhages and blood cell infiltration in the gastric mucosa [43,44]. The report of ulcer reduction by *Ceiba pentandra* leaves in this study corroborates earlier reports of the anti-ulcerogenic activity of *Ceiba pentandra* against indomethacin-induced ulcer [21-22].

In the diabetes study, there were significant elevations in the activities of the liver enzymes (ALP, ALT and AST) and lipid profiles (cholesterol, TAG and LDL) in the diabetes-untreated rats when compared to those of the non-diabetic (normal) control rats. Mild chronic elevations of transaminases are frequently found in type 2 diabetic patients and often reflect underlying insulin resistance. Potential explanations for elevated transaminases in insulin-resistant states include oxidant stress from reactive lipid peroxidation, peroxisomal beta-oxidation, and recruited inflammatory cells [16,45]. The insulin-resistant state is also characterized by an increase in proinflammatory cytokines such as tumor necrosis factor-α (TNF- α), which may also contribute to hepatocellular injury. The liver helps maintain normal blood glucose concentration in the fasting and postprandial states. Loss of insulin effect on the liver leads to glycogenolysis and an increase in hepatic glucose production [46-47]. In contrast, anti-diabetic agents decrease the aminotransferase levels as tighter blood glucose levels are achieved. Treatment of the animals with 200 and 400 mg/kg of the methanol extract of *Ceiba pentandra* leaves produced significant dose-dependent decreases (P<0.05) in the liver enzymes with values comparable to those of glibenclamide-treated and normal control groups. Diabetes mellitus is also characterized by chronic hyperglycaemia and lipoprotein abnormalities. This is as a result of altered intermediary regulation of major food substrates. Deficiency of a polypeptide hormone, insulin, which plays a role in the metabolism of carbohydrate, is a predisposing factor leading to hypercholesterolaemia and hypertriglyceridaemia [48]. Abnormalities of triglyceride storage and lipolysis in insulin-sensitive tissue such as the liver are also an early manifestation of diabetes characterized by insulin resistance [49]. Insulin deficiency or resistance could be responsible for dyslipidaemia because it increases fatty acid as well as triglyceride synthesis in adipose tissue and liver [50] In severe and uncontrolled diabetes, the resultant increases in LDL, triglycerides and total cholesterol are associated with increased morbidity and mortality from coronary artery disease [51]. Treatment with methanol extract of *Ceiba pentandra* leaves in this study significantly decreased the elevated levels of blood glucose, serum cholesterol, triacylglycerides, and LDL and increased the level of HDL in the alloxan-induced diabetic rats. Earlier studies have reported the hypoglycaemic activity of *Ceiba pentandra* stem bark and leaves on alloxan and Streptozotocin -induced diabetic rats [20,52]. Effective control of blood glucose is a key step in preventing diabetic complications and

improving the quality of life in both Type I and Type II diabetic patients [53-54]. Reports have shown that most plants used in the treatment and management of diabetes possess both hypoglycaemic and anti-oxidative properties [55-57]. Numerous mechanism of action have been proposed for these plant extracts. Some hypotheses relate their effect to the activity of pancreatic β cells (synthesis, release, cell regeneration) [58] the increase in the protective/inhibitory effect against insulinase and increase of insulin sensitivity or the insulin-like activity of the plant extracts [59], inhibition of renal glucose reabsorption [58] and inhibition of endogenous glucose production [60]. These actions may be responsible for the reduction or abolition of the diabetic complications. Reduction of blood glucose by *Ceiba pentandra* leaves in this study could perhaps be related to stimulation of beta cells of Islet of Langerhans to enhance insulin secretion or increase of the body's sensitivity for insulin. Significant elevation of HDL in the *Ceiba pentandra* extract treated groups suggests that the plant has protective effect on the heart. The phytochemical results of *Ceiba pentandra* leaves showed the presence of flavonoids, glycosides, alkaloids, saponnins and tannins. Some of these plant components, particularly flavonoids and tannins, have been reported to have hypoglycaemic activity [61]. It is therefore speculated that the hypoglycaemic effect exhibited by methanol extract of *Ceiba pentandra* leaves in the present study may partly be due to some of these bioactive components. The result from this study showed that the methanol extract of *Ceiba pentandra* leaves decreased the activities of the liver enzymes, fasting blood glucose and lipid profile levels in diabetic rats and thus, could be effective in the management of diabetes and its complications.

5. CONCLUSION

The results shown in this work suggest that the methanol extract of *Ceiba pentandra* leaves has potential for use in the treatment of stomach ulcers and management of diabetes mellitus and its associated complications.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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An Overview on Nano Drug Delivery: Challenges and Its Safety Issues

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ABSTRACT

Nanotechnology is the science and technology of precisely manipulating the structure of matter at the molecular level. It is the use and manipulation of matter at a tiny scale. At this size, atoms and molecules work differently, and provide a variety of surprising and interesting uses. Nanotechnology deals with the creation of useful materials, device and systems and systems through control of matter on the nanometer length scale and exploitation of novel phenomena and properties at that length scale. Nanotechnology is one approach to overcome challenges of conventional drug delivery systems based on the development and fabrication of nanostructures. Various nanostructures employed in drug delivery, their methods of fabrication and challenges of nano drug delivery are reviewed. The present subject matter is mainly conferred on different nanostructures and its challenges and possible safety issues. Some challenges associated with the technology as it relates to drug effectiveness, toxicity, stability and drug regulatory control. It is anticipated that better understanding and application of nanotechnology for effective drug delivery would ultimately enhance efficacy of treatment and patient compliance in drug use. It would be difficult to refute the potential benefits of nanotechnology and stop development of research related to it since it has already begun to break through many different fields of research. However, nanotechnology can be developed using guidelines to assure that the technology does not become too potentially harmful.

Keywords: Nanotechnology; nanoparticles; solid lipid nanoparticles; nano-emulsion; drug delivery; nanomaterials.

1. INTRODUCTION

With advancements in nano science and technology, a large number of materials and improved products may be available with a change in the physical properties when their sizes are shrunk. Nanotechnology-based delivery systems can also protect drugs from degradation. Nanotechnology is shown to bridge the barrier of biological and physical sciences by applying nanostructures and nanophases at various fields of science [1]; specially in nanomedicine and nano based drug delivery systems, where such particles are of major interest [2,3,4]. These properties can help reduce the number of doses required, make treatment a better experience and reduce treatment expenses. A number of nano-based systems allow delivery of insoluble drugs, allowing the use of previously rejected drugs or drugs which are difficult to administer e.g. paclitaxel.The fabrication of nanostructures is able to provide superior drug delivery systems for better management and treatment of diseases. The first fabrication of nanoparticles was about 35 years ago as carriers for vaccines and cancer chemotherapeutics [5,6]. The nanostructures employed as drug delivery systems have multiple advantages which make them superior to conventional delivery systems. The benefits account for the extensive research that have been undertaken into the development of nanostructures such as liposomes, nanocapsules, nanoemulsions, solid lipid nanoparticles, dendrimers, polymeric nanoparticles, etc, for delivery of drugs. The materials employed in the fabrication of nanostructures determine the type of nanostructures obtained and these nanostructures, in turn, determine the different properties obtained and the release characteristics of incorporated drugs. The therapeutic

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value of many promising drugs for the treatment of various neurological disorders is diminished by the presence of the blood-brain barrier. The blood-brain barrier is a distinctive membrane that tightly segregates the brain from the circulating blood. Thus, drug delivery to this organ is a challenge, because the brain benefits from very efficient protection.

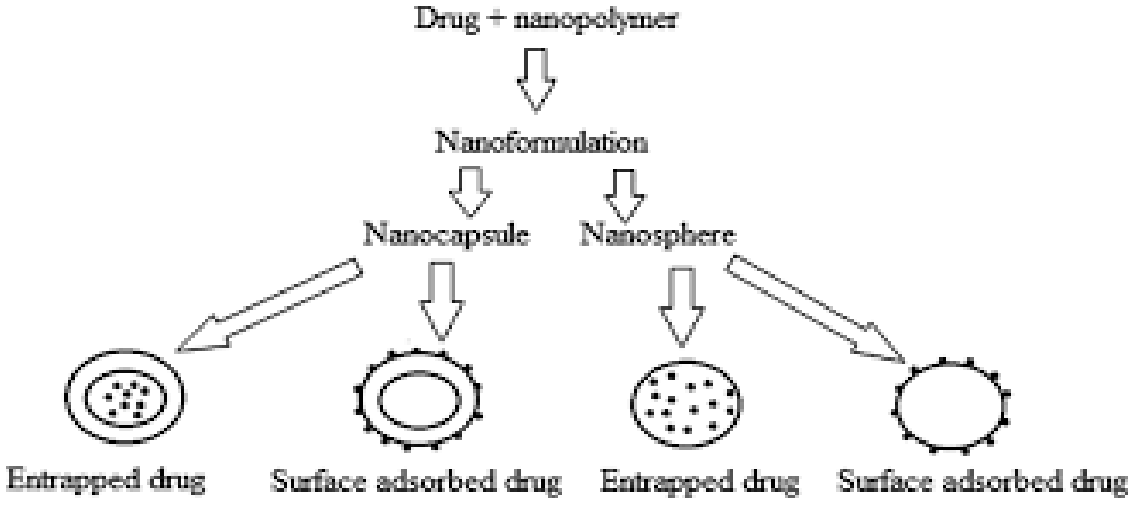
Nanotechnology offers a solution for using the copious chemical entities for treating brain disorders that are not clinically useful because of the presence of the blood-brain barrier. Nano-particles can be effectively used to deliver relevant drugs to the brain.

2. TYPES OF NANO-STRUCTURES

2.1 Polymeric Nano-particles

Polymeric nanoparticles are colloidal solid particles with a size range of 10 to 1000nm and they can be spherical, branched or shell structures. The first fabrication of nanoparticles was about 35 years ago as carriers for vaccines and cancer chemotherapeutics. They are developed from non-biodegradable and biodegradable polymers. Their small sizes enable them to penetrate capillaries and to be taken up by cells, thereby increasing the accumulation of drugs at target sites. Drugs are incorporated into nanoparticles by dissolution, entrapment, adsorption, attachment or by encapsulation and the nanoparticles provide sustained release of the drugs for longer periods, e.g., days and weeks. Nanoparticles enhance immunization by prevention of degradation of the vaccine and increased uptake by immune cells. One of the determinants of the extent of uptake by immune cells is the type of polymer employed [7,8].

Some polymers used in the fabrication of nanoparticles include chitosan, alginate, albumin, gelatin, polyacrylates, polycaprolactones, poly(D, L-lactide-co-glycolide) and poly (D, L-lactide).



(Polymeric Nano-particle as carrier)

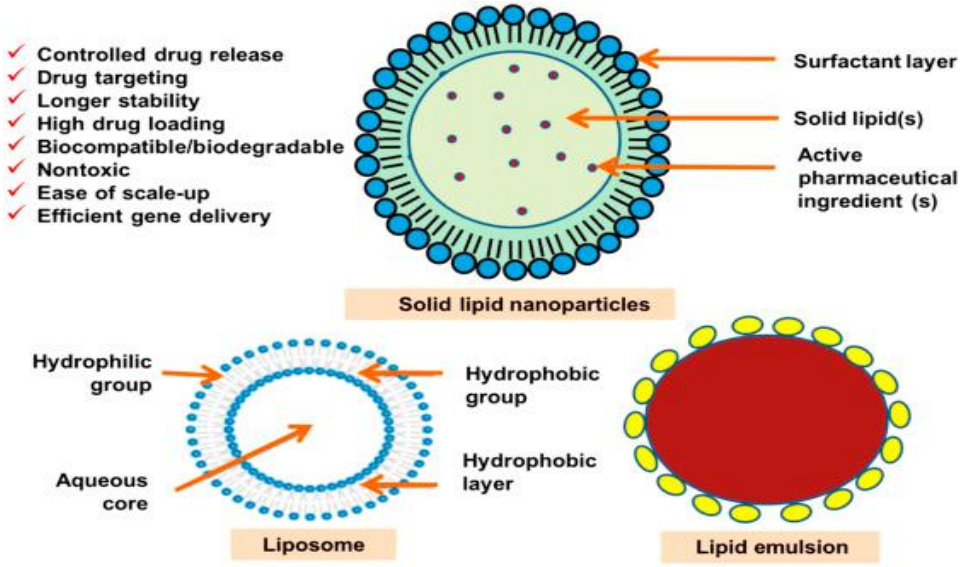
2.2 Liposomes

Liposomes were first developed about 40 years ago. They are small artificial vesicles (50 – 100 nm) developed from phospholipidssuch as phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylserine, which have been used in biology, biochemistry, medicine, food and cosmetics. The characteristics of liposomes are determined by the choice of lipid, their composition, method of preparation, size and surface charge. Liposomes have been applied as drug carriers due to their ability to prevent degradation of drugs, reduce side effects and target drugs to site of action. However, limitations of liposomes include low encapsulation efficiency, rapid leakage of water-soluble drug in the presence of blood components and poor storage stability. Applications of

liposomes include transdermal drug delivery to enhance skin permeation of drugs with high molecular weight and poor water solubility; drug delivery to the lungs by nebulisation; ocular drug delivery and in the treatment of parasitic infections.

2.3 Solid Lipid Nanocarriers

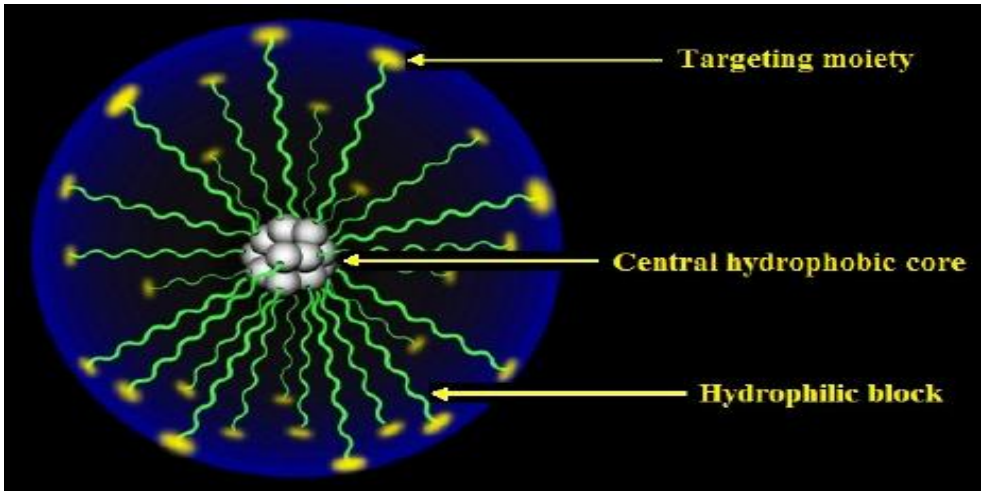
Solid lipid nanoparticles (SLN) are nanostructures made from solid lipids such as glyceryl behenate (Compritol), stearic triglyceride (tristearin), cetyl palmitate and glycerol tripalmitate (tripalmitin) with a size range of 50 and 1000 nm. Research interest in SLN emerged about ten years ago due to their scalability potential. The lipids employed are well tolerated by the body. Large scale production will be cost effective and simple by using high pressure homogenization. Some of the features of SLN include good tolerability, site-specific targeting, stability (stabilized by surfactants or polymers), controlled drug release and protection of liable drugs from degradation. However, SLN are known for insufficient drug loading, drug expulsion after polymorphic transition on storage and relative high water content of the dispersions. SLN has been studied and developed for parenteral, dermal, ocular, oral, pulmonary and rectal routes of administration [9].



2.4 Polymeric Micelles

Micelles are formed when amphiphilic surfactant or polymeric molecules spontaneously associate in aqueous medium to form core-shell structures or vesicles. Polymeric micelles are formed from amphiphilic block copolymers, such as poly (ethylene oxide)-poly(-benzyl-Laspartate) and poly(N-isopropylacrylamide)-polystyrene, and are more stable than surfactant micelles in physiological solutions. They were first proposed as drug carriers about 25years ago. The inner core of a micelle is hydrophobic which is surrounded by a shell of hydrophilic polymers such as poly (ethylene glycol). Their hydrophobic core enables incorporation of poorly water soluble and amphiphilic drugs while their hydrophilic shell and size (<100nm) prolong their circulation time in the blood and increase accumulation in tumoural tissues [10].

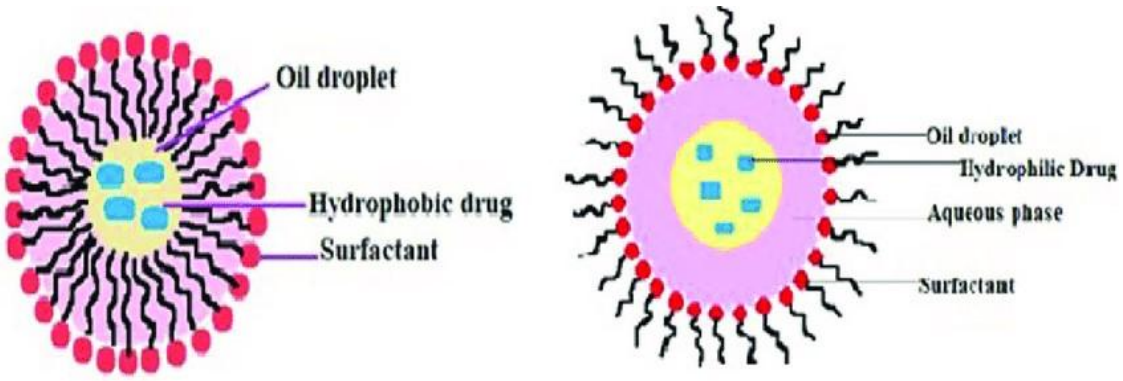
Polymeric micelles are able to reach parts of the body that are poorly accessible to liposomes; accumulate more than free drugs in tumoral tissues due to increased vascular permeability. Thus, polymeric micelles can be employed to administer chemotherapeutics in a controlled and targeted manner with high concentration in the tumoural cells and reduced side effects. Polymeric micelles have been employed for targeted and intracellular delivery, sustained release and parenteral delivery.



(Structure of the Polymeric micelles)

2.5 Nanoemulsions

Nanoemulsions are emulsions with droplet size below 1µ but usually between 20 and 200 nm. Unlike microemulsions which are white in colour due to their light scattering ability, nanoemulsions whose nanosize is often smaller than visible wavelength, are transparent. Nanoemulsions are biodegradable, biocompatible, easy to produce and used as carriers for lipophilic drugs which are prone to hydrolysis. They are employed as a sustained release delivery system for depot formation via subcutaneous injection. They enhance gastrointestinal absorption and reduce inter- and intra-subject variability for various drugs. Due to their very large interfacial area, they exhibit excellent drug release profile. Stability against sedimentation is attained based on the nano size of the droplets because the sedimentation rate due to gravity is less than Brownian movement and diffusion. Unlike microemulsions, nanoemulsions are metastable and can be destabilized by Ostwald ripening whereby the small droplets dissolve and their mass is taken up by the large droplets and depletion induced flocculation due to addition of thickening polymers. When this happens, the nanoemulsion becomes opaque and creaming will occur. However, addition of a small amount of second oil with low solubility into the aqueous phase and addition of a second surfactant may reduce Ostwald ripening [11].



(Nanoemulsion (O/W and W/O Type))

2.6 Metallic Nanoparticles

Metallic nanoparticles include iron oxide, gold, silver, gadolinium and nickel which have been studied for targeted cellular delivery. Gold exhibits favourable optical and chemical properties at nanoscale for

biomedical imaging and therapeutic applications. It can be manipulated to obtain the desired size in the range of 0.8 to 200 nm. The surface can be modified with different functional groups for gene transfection, modified into gene delivery vector by conjugation and also modified to target proteins and peptides to the cell nucleus. Gadolinium has been studied for enhanced tumour targeted delivery by modification of the nanoparticles with folate, thiamine and poly (ethyleneglycol). Metallic nanoparticles have large surface area thereby incorporating a high drug dose. However, the toxicity of metallic nanoparticles is of concern.

2.7 Dendrimers

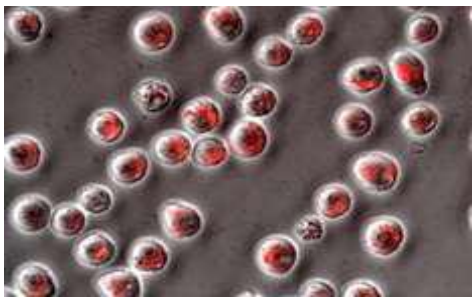
Dendrimers are nanostructures produced from macromolecules such as polyamidoamine (PAMAM), polypropyleneimine and polyaryl ether; and are highly branched with an inner core. The particle size range is between 1 to 100nm although their sizes are mostly less than 10nm. About 20 years ago, dendrimer studies centred on their synthesis, physical and chemical properties while exploration of their biological applications was initiated about thirteen years ago. The uniqueness of dendrimers is based on their series of branches, multivalency, well defined molecular weight and globular structure with controlled surface functionality, which enhances their potential as carriers for drug delivery. Dendrimers have been reported to provide controlled release from the inner core. However, drugs are incorporated both in the interior as well as attached on the surface. Due to their versatility, both hydrophilic and hydrophobic drugs can be incorporated into dendrimers.

2.8 Nanocapsules

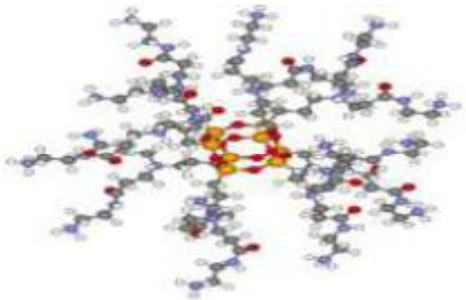
Nanocapsules are spherical hollow structures in which the drug is confined in the cavity and is surrounded by a polymer membrane. They were developed over 30 years ago. Sizes between 50 and 300nm are preferred for drug delivery and they may be filled with oil which can dissolve lipophilic drugs. They have low density, high loading capacity and are taken up by the mononuclear phagocyte system, and accumulate at target organs such as liver and spleen. Nanocapsules can be employed as confined reaction vessels, protective shell for cells or enzymes, transfection vectors in gene therapy, dye dispersants, carriers in heterogenous catalysis, imaging and drug carrier. Encapsulation of drugs such as ibuprofen within nanocapsules protects liable drugs from degradation, reduces systemic toxicity, provide controlled release and mask unpleasant taste [12,13].

2.9 Carbon Nanomaterials

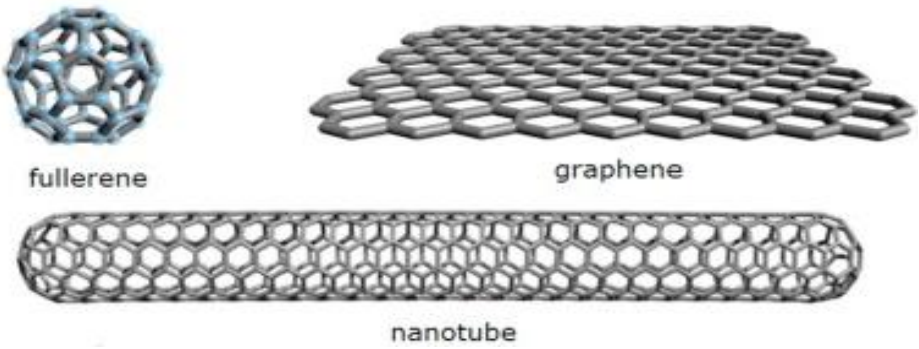
These include carbon nanotubes and fullerenes. Fullerenes are carbon allotrope made up of 60 or more carbon atoms with a polygonal structure. Nanotubes have been used for their high electrical conductivity and excellent strength. These materials are being studied for therapeutic applications.Fullerenes can be functionalized for delivery of drugs and biomolecules across cell membrane to the mitochondria. Carbon nanotubes' unique properties including low cytotoxicity and good biocompatibility attract their use as vector system in target delivery of drugs, proteins and genes. However, toxicity of carbon nanotubes is of great concern. Carbon nanotubes may cause inflammatory and fibrotic reactions [14].



(Nano-Capsule)



(Dendrimers)



(Carbon nanomaterials)

3. CHALLENGES AND SAFETY ISSUES

Although nanotechnology in drug delivery has been successful, as evidenced by some nano drug products in the market, not all approaches have met with the same success. New nanomaterials being developed come with challenges, which have to be overcomed. However some of the challenges encountered have been and are still being tackled by modification of the physicochemical characteristics of the nanomaterials to improve on properties such as long circulation in the blood, increased functional surface area, protection of incorporated drug from degradation, crossing of biological barriers and site-specific targeting [15,16].

3.1 Manufacturing Issue

Another challenge of research and development (R&D) of nanomaterials for drug delivery is large scale production. There is always a need to scale up laboratory or pilot technologies for eventual commercialization. A number of nano drug delivery technologies may not be scalable due to the method and process of production and high cost of materials employed. it is easier to modify nanomaterials at laboratory scale for improved performance than at large scale. Maintaining the size and composition of nanomaterials at large scale is also a challenge. Despite the number of patents for nano drug delivery technologies, commercialization is still at its early stage. This is partially because researchers in academia carry out most of the research studies in nano drug delivery. Therefore, for these technologies to get to the market there has to be increased partnership with the pharmaceutical companies. Unfortunately, a number of the major pharmaceutical industries are yet to consider nanotechnology as one of their priorities due to lack of regulatory guidelines and challenges of scaling up. . However, it is envisaged that with the expiration of more patents and market loss, more pharmaceutical industries will take up the production of nano drug products in order to compete favorably. Advances in nano drug delivery technology also provide new challenges for regulatory control. There is an increasing need to have regulations that would account for physicochemical and pharmacokinetic properties of nano drug products, which are different from conventional drug products. The United States' Food and Drug Administration (FDA) and the European Medicines Evaluation Agency (EMEA) have taken the initiative to identify some possible scientific and regulatory challenges. Furthermore, the International Organization for Standardization has set up a technical committee (TC 229) for the field of nanotechnologies to develop standards pertaining to terminology and nomenclature; measurement and characterization; and health, safety and environment amongst other standards. These standards are still under development.

Another challenge facing nanodrug delivery is the large-scale production of nanomaterials in terms of scaling up laboratory or pilot technologies for consistent and reproducible production and commercialization. The challenges of scaling up include a low concentration of nanomaterials, agglomeration and the chemistry process. It is much easier to modify or maintain the size or composition of nanomaterials at the laboratory scale for improved performance than at a large scale. The biomedical community should re-think the level of control needed when working with nanomaterials. Rather than requiring perfect control of the physical dimensions of nanomaterials, a

statistical approach may be adopted in order to establish a metric for classifying nanomaterials by material type, average size, aspect ratio and standard deviation. This would fit well with the formation of a toxicology database, since it is unrealistic to establish the toxicology of every size or aspect ratio of a nanomaterial.

3.2 Safety Issues

Nanoparticles can be inhaled, ingested or absorbed through the skin, and they can penetrate cells, even into the cell nucleus, where, if sufficiently small, they can come into close contact with genetic material. Thus, nanomaterial toxicity should be considered relative to the patient population, as well as the entire manufacturing and disposal processes. Based on safety concerns, the establishment of standards or reference materials and consensus testing protocols that can provide benchmarks for the development of novel classes of materials are needed. It is not clear as to how they cause lung injury, but a recent study published in the Journal of Molecular Cell Biology showed that Polyamine dendrimers (PAMAM's) trigger a programmed cell death called as autophagic cell death thereby causing lung damage. Autophagy is a normal cell scavenging process. It disintegrates damaged cells and regulates normal cell growth. Over-activity of this process leads to death of lung cells, leading to organ damage. It is not confirmed whether other group of nanoparticles (apart from PAMAM's) work by the same mechanism but a few may do so and blocking autophagic cell death can lead to prevention of lung damage in most cases .Pulmonary inflammation can also lead to changes in membrane permeability that may cause the nanoparticles to distribute beyond the lungs. Other than the evident risks to the patient, nanoparticles may perhaps be toxic to the environment too, and may require prior processing before disposal. The non-biodegradable ones are likely to cause land, water or air pollution. It is tough to predict their effects on the surroundings and, it is not known whether or not they are harmful to the biomass. If they enter the bionetwork through the plants, their eradication would be highly demanding.

Conventional safety measures in a pharmaceutical factory may not be appropriate for the development and fabrication of nanomaterials. Also extra measures are to be taken to protect the environment from increased envisaged negative impacts of nanomaterials. Although reduced cost to the patients is envisaged to be one of the advantages of nanotechnology since fewer materials are expected to go into production as compared to bulk production, it is doubtful if this will be so, as successful commercialization will be expensive.

With increased R&D work on nano drug delivery, emerge concerns about the safety of the nanotechnologies in humans. Some of the nanomaterials are biodegradable while some are not; furthermore, the side effects of the by-products present a huge concern. Materials which may be safe at macroscale may not be at nanoscale since there may be change in physicochemical characteristics at nanoscale. These nanomaterials may not clear completely from the body and their accumulation may have several possible effects. Safety and possible impact nanomaterials should not be considered for the patient population alone but also for the entire manufacturing and disposal processes.

4. CONCLUSION

There is the general public hesitation to cuddle nanotechnology based on the unavailability of documented safety guidelines. However, in spite of these challenges, nano drug delivery is a development that cannot be ignored and so the challenges will be embarking upon with time.

The increasing awareness of R&D in the area of nano drug delivery would continue to change the whole concept of medicines including aspects such as product characteristics, bioavailability, pharmacokinetics, stability, drug use, and toxicity in human as well as animal and plant diseases. This in itself poses enormous challenges to the formulation scientist who has to keep abreast of rapid developments in this field. A whole segment of R & D has opened up, posing great challenges to equipment manufacturers, material scientists, pharmaceutical researchers and regulatory agencies. It is anticipated that better understanding and application of nanotechnology for effective drug delivery would ultimately enhance efficacy of treatment and patient compliance in drug use. It would be difficult

to disprove the potential benefits of nanotechnology and stop development of research related to it since it has already begun to break through many different fields of research. However, nanotechnology can be developed using guidelines to assure that the technology does not become too potentially harmful to the mankind.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 10
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Glycemic Index Profiling of Raw and Germinated Quinoa (*Chenopodium quinoa* Willd)

W. Jessie Suneetha[1*] and B. Anila Kumari[2]

DOI: 10.9734/bpi/tprd/v5

ABSTRACT

Quinoa has proteins of high biological value, carbohydrates of low glycemic index and phytosteroids with ω - 3 and 6 fatty acids that provide health benefits. The main advantage of quinoa is that along with being a protein supplement in the flour industry, it meets the increasing international demand for gluten-free products in cakes, pastries, pasta and baked goods. Studies on glycemic index (GI) profiling of newly released quinoa varieties are lacking and so the present study was taken up to assess its GI. Cooked raw and germinated quinoa were given to 10 subjects, their blood glucose levels were determined every 15 min up to 60 min and again at 90 and 120 min respectively. The blood glucose levels after consumption of raw quinoa ranged from 78.30 to 120.20 mg/dl with an average fasting level of 96.51 mg/dl and for germinated quinoa ranged from 68.4 to 114.0 mg/dl with an average fasting level of 84.2 mg/dl. The glycemic index of raw quinoa of 70 g having 50 g of carbohydrates ranged from 51.28 to 78.25 with an average of 63.37 and the glycemic load was from 25.64 to 39.12 with an average of 31.68. The glycemic index of germinated quinoa of 75 g having 50 g of carbohydrates ranged from 51.2 to 64.7 with an average of 59.0 and the glycemic load was from 25.6 to 29.7 with an average of 29.54. Hence, this quinoa variety can be categorized as a medium glycemic index and high glycemic load food. These diets can reduce insulin resistance and the risk of life style diseases like CVD, diabetes and certain cancers.

Keywords: Quinoa; gluten-free; germination, glycemic index and glycemic load.

1. INTRODUCTION

Glycemic index (GI) describes the blood glucose response after consumption of carbohydrate-containing test food relative to a carbohydrate-containing reference food, typically glucose. GI helps people with diabetes and obesity in the selection of low GI foods. Although Otto et al. [1,2] first brought attention to the different glycemic effects of various foods, the glycemic index (GI) was initially conceived by Jenkins et al. [3] as a tool for the dietary management of type 1 diabetes and, later, dyslipidemia [4,5]. The foods with low GI or GL benefit by controlling blood glucose levels in diabetics along with lipid management. At times, low GI and GL foods can be energy dense with substantial amounts of sugars or undesirable fats that contribute to a diminished glycemic response there by questioning the health claims [6]. The appearance of glucose in the bloodstream following eating—the glycemic response (GR)—is a normal physiological occurrence that depends on the rate of glucose entry into the circulation, the amount of glucose absorbed, the rate of disappearance from the circulation due to tissue uptake, and hepatic regulation of glucose release [7,8].

The GI is the ranking of carbohydrates on a scale of 0 to 100 as per their impact on blood sugar levels during the 2 hrs following the consumption of food. Low GI foods produce a gradual rise in blood sugar levels and are considered healthy particularly in the prevention of life style diseases [9]. They regulate lipid profile and help in weight management by controlling appetite due to slow gastric

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emptying. These diets reduce insulin resistance by improving blood glucose levels along with the risk of obesity, cardiovascular diseases, diabetes and certain types of cancers [10].

Quinoa carbohydrates can be used as nutraceuticals due to its hypoglycemic effect, reduction of free fatty acids and triglycerides. Studies in individuals with celiac disease showed that the GI of quinoa was comparatively lower than the common gluten free pasta and bread [11].

Low GI foods are associated with a greater feeling of satiety due to delayed emptying of the stomach. The foods rich in dietary fibre can induce a low glycemic response and prolong gastric distension increasing the peptides associated with satiety. The whole grain pasta showed lower glycemic response than the refined grain pasta with greater satiety [12].

Quinoa milk can be consumed directly or in milky products in near future due to its proteins with high biological value, carbohydrates of low glycemic index and phytosteroids with ω - 3 and 6 fatty acids. It can be consumed by people who are lactose or casein intolerant [13]. Quinoa milk has low GI than rice milk due to its complete protein content which slows down the digestion and gastric emptying [14].

Few studies on the glycemic index of quinoa grain and its products are available and hence the present study was under taken for glycemic index profiling along with popularization in this rain-fed region.

2. MATERIALS AND METHODS

Preparation of sample: The quinoa variety analysed was one of the accession lines of EC series brought from Peru and tested at Agricultural Research Station, Anantapur. Raw quinoa seeds were cleaned; washed 5 to 6 times until no frothing appeared to remove the saponins and pressure cooked with 3 times the water.

The germination of quinoa seeds was carried out by soaking in distilled water for 10 hrs, spread on wet laboratory paper in trays and covered with the same wet paper to hydrate them by capillarity. These trays were incubated at 20°C in BOD chamber for 4hrs. Then the samples were taken and dried at 50°C in a tray drier to constant weight. Dried sprouts were stored in plastic bags in desiccators at 4°C [15].

Selection of subjects: Initially fifteen college-going students from D hostel annex of PJTSAU campus in the age group of 19-22 years were selected. Subjects were excluded if they reported any history of gastrointestinal disorders or were taking medication for any chronic disease conditions or intolerant or allergic to any of the foods. Finally, 10 healthy subjects were identified for study from initial 15 members and consent taken from the subjects to proceed further with the study. Before starting the GI trial, subjects were given necessary instructions about the study. This study was sent to the university for approval and the Endt. No. B-148/PG/A2/2015 and Endt. No. B-136/PG/2015 were given to proceed further.

Glycemic index (GI) profiling: The method used for measuring and calculating the GI of the quinoa was in accordance with WHO/FAO recommendations [16]. Subjects attended each testing session after 10 hrs overnight fast but not exceeding 16 hrs and were informed not to consume unusually large meals and exercise vigorously on the previous night. On the first occasion, the subjects were given the standard reference food which is usually 'glucose'. Glucose of 50.0 g was added to 250 ml water and given to subjects as a drink. Next day, 70.0 g of cooked test quinoa containing 50.0 g of carbohydrates was given to each subject. On both the occasions, blood glucose levels were measured by using one-touch glucometer in capillary whole blood obtained by finger prick at 15, 30, 45, 60, 90 and 120 min after consumption.

Determination of GI and glycemic load (GL): The incremental area under 2-hour glucose response curve (IAUC) was calculated [17]. The calculations of GI and GL were as follows:

$$GI = \frac{\text{IAUC of test food}}{\text{IAUC of reference food}} X100$$

$$GL = \frac{GI}{100} \text{ X Dietary carbohydrate content of test sample}$$

The relationship between GI and GL is not straightforward. A high GI food can have a low GL if eaten in small quantities and a low GI food can have a high GL if eaten in bulk. The GI and GL are dependent upon the portion size eaten and on the carbohydrate content of the foods taken [18].

3. RESULTS AND DISCUSSION

The fasting insulin is lower in individuals with higher dietary fibre intake and the ingestion of complex carbohydrates promotes longevity. Glucose is used as reference food and is rated as 100. As per GI classification, high GI foods have values > 70, medium GI foods have values between 56 - 69 and low GI foods have values ≤ 55 [19]. The GI profiling of cooked raw and germinated quinoa in this sent study were presented in Table 1 and Table 2 with the mean scores of blood glucose levels in Fig. 1 and Fig. 2.

Table 1. Glycemic index and glycemic load of raw quinoa [24]

S. No.	GI	GL
1.	56.11	28.05
2.	78.25	39.12
3.	60.11	30.05
4.	65.72	32.86
5.	57.61	28.80
6.	51.28	25.64
7.	67.32	33.66
8.	74.71	37.35
9.	56.53	28.26
10.	66.08	33.04
Mean	63.37	31.68

Note: Values for ten subjects shown in the table.
GI - Glycemic index; GL - Glycemic load

Table 2. Glycemic index and glycemic load of germinated quinoa [25]

S. No.	GI	GL
1.	54.9	27.45
2.	64.7	32.35
3.	51.2	25.60
4.	55.5	27.75
5.	56.3	28.15
6.	57.6	28.80
7.	82.5	41.25
8.	59.4	29.70
9.	53.6	26.80
10.	55.2	27.59
Mean	59.08	29.54

Note: Values for ten subjects shown in the table.
GI - Glycemic index
GL - Glycemic load

It was observed that the glucose levels were lower initially at 83.90 mg/dl for raw quinoa and 84.2 mg/dl for germinated quinoa as the subjects have not taken any food for at least the last 10 hrs before the test. But 15 min after consumption of raw and germinated cooked quinoa, there was an increase in blood glucose level to 120.20 mg/dl and 114.0 mg/dl whereas 2 hrs after consumption it gradually lowered to 78.30 mg/dl and 68.4 mg/dl indicating that slow release into the blood.

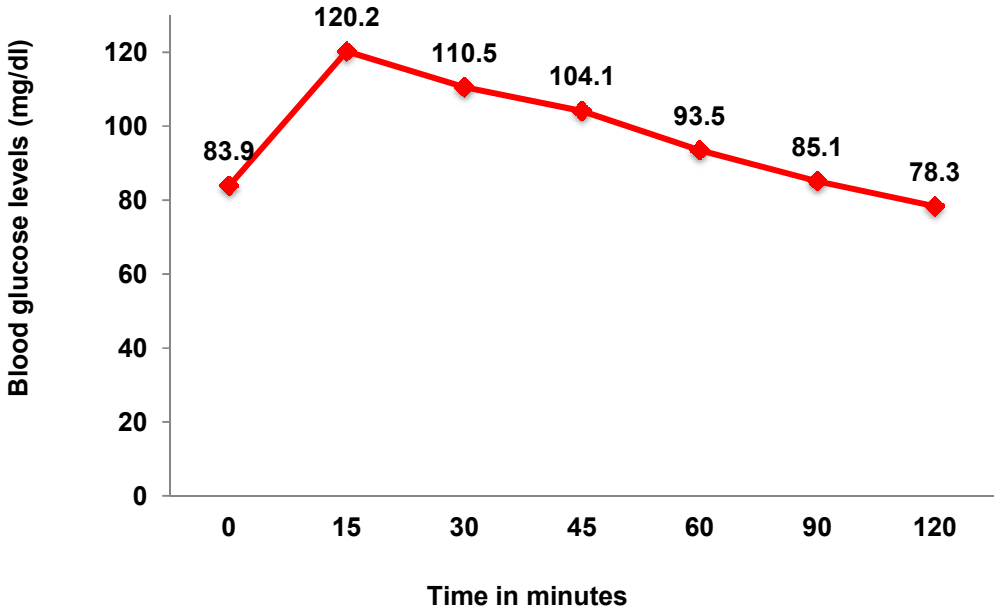

Fig. 1. Mean blood sugar levels after consumption of raw cooked quinoa [24]

The mean fasting blood glucose level was 96.51 mg/dl and 84.2 mg/dl for raw and germinated quinoa. The blood glucose levels after consumption of raw cooked quinoa ranged from 78.30 to 120.20 mg/dl for 70 g containing 50 g of carbohydrates whereas for germinated cooked quinoa ranged from 51.2 to 64.7 with an average of 59.0 for 75 g having 50 g of carbohydrates.

The GI ranged from 51.28 to 78.25 with an average of 63.37 for 100g of raw cooked quinoa and for germinated quinoa ranged from 68.4 to 114.0 mg/dl with an average of 84.2 mg/dl. Results showed that quinoa can be classified under medium GI food [19]. The glycemic load of quinoa ranged from 25.64 to 39.12 with an average of 31.68 for cooked raw quinoa whereas for cooked germinated quinoa ranged from 25.6 to 29.7 with an average of 29.54 and were classified as high GL food as per [20]. The high GL foods have values ≥20, medium GL foods have values between 11-29 and low GL foods have values ≤ 10.

Recent studies have shown the ability of lower GI meals to help improve glycemic levels in diabetics. Studies in healthy individuals, adults with type II diabetes and youth with type I diabetes has shown that the use of GI methodology in the selection of carbohydrates may have beneficial implications on blood glucose responses. The GI concept may be of particular benefit to Indians as the incidences of impaired glucose tolerance and diabetes are on the raise [21].

GI of unprocessed quinoa between 35.0 – 53.0. When compared with the study, GI values were more indicating that germination will increase GI values due to breakdown of carbohydrates [19].

Another study showed that daily consumption of 50.0 g of gluten-free quinoa over a six-week period was safely tolerated by celiac patients without any deterioration in health condition and with improved histological and serological parameters along with mild hypocholesterolemic effect [22].

The low GI diets help in the prevention of diabetes and coronary heart diseases [23]. The subjects with type 2 diabetes showed improved metabolic control when the conventional high GI breakfast was replaced with low GI meal [19].

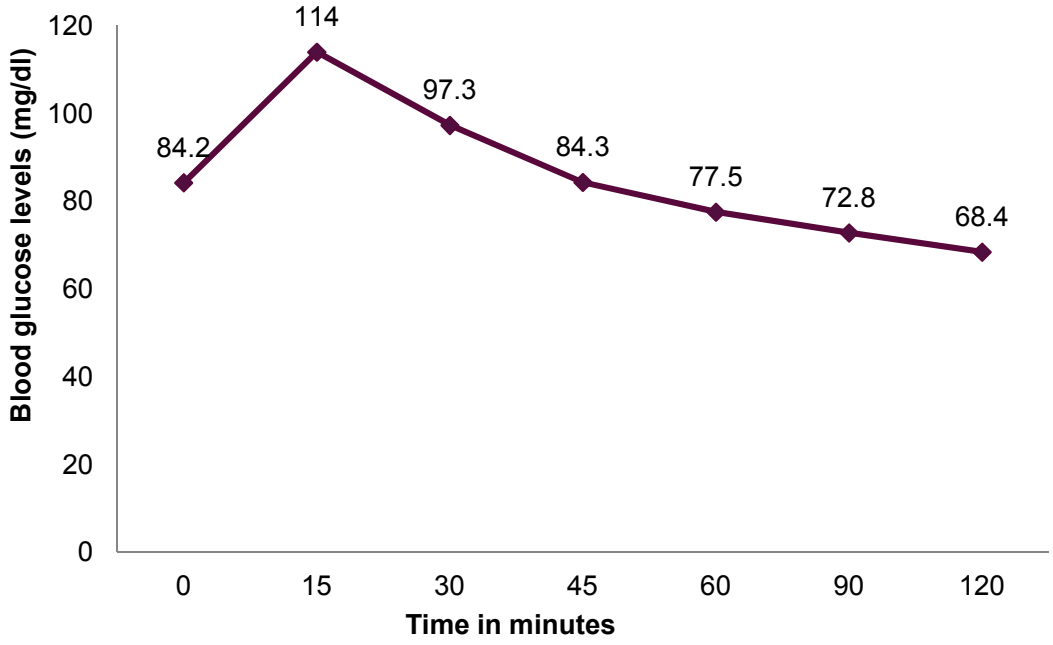

Fig. 2. Mean blood sugar levels after consumption of germinated cooked quinoa [25]

4. CONCLUSION

The GI provides a good summary of postprandial glycemia. Low GI diets have been shown to improve glucose levels, regulate lipid profile, remove free fatty acids and control weight gain as they help in managing appetite by extending gastric emptying. These diets can reduce insulin resistance and the risk of life style diseases like CVD, diabetes and certain cancers.

CONSENT

As per University procedure, subject's oral consent has been taken by the authors before the start of study.

ETHICAL APPROVAL

As per University procedure, approval of for the study was taken by the authors (Endt. No. B-148/PG/A2/2015 and Endt. No. B-136/PG/2015).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 11
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Highlighting New Antiretrovirals on the Block: Pharmacological News from CROI 2017

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ABSTRACT

The Conference on Retrovirus and Opportunistic Infections, CROI, 2017 at Seattle, USA, presented several new substances for the treatment of HIV/AIDS. This review shows the most promising candidates and discusses the mechanism of action, safety profile and potential drug-drug-interactions of new integrase strand inhibitors (INSTI), nucleoside (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) as well as protease inhibitors (PI), CCR5-inhibitors, long-acting antibodies or *nano particle* PI and NNRTI (NANO-NNRTI, NANO-PI).

Keywords: New antiretrovirals; HIV-therapy; drug-drug-interactions.

1. INTRODUCTION

The Conference on Retrovirus and Opportunistic Infections, CROI, 2017 at Seattle, USA, presented several new substances, therapy strategies and other data about the treatment of HIV/AIDS. The following article discusses a pharmacological selection of these and shows data of new integrase inhibitors (INSTI), nucleoside (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) as well as protease inhibitors (PI), CCR5-inhibitors and several long-acting antibodies or new formulations of already widely used drugs, such as *nano particle* PI and NNRTI (NANO-NNRTI, NANO-PI). The triple therapy has resulted into significant improvement in an immune status, quality of life, and reduced morbidity and mortality associated with HIV infection [1,2].

2. NUKLEOS(T)IDE REVERSE TRANSKRIPTASE INHIBITORS

Karen White and colleagues presented the new NRTI GS-9131, which reveals strong activity against NRTI-resistant HIV-1.

At a very low EC_{50} of only 0.16 µM (±0.02) GS-9131 is still active against most of NRTI-resistant HIV-1, showing mutations of K65R, M184V, L74V/I, 6TAMs+184V or Q151M+M184V.

Unlike TAF, GS-3131 is an adenosine-analogue. But alike TAF it is a prodrug, which is modified by cathepsin A into its intracellular form of GS-9148 and then phosphorylated by intracellular kinases into its antiretroviral active diphosphate.

Additive effects are seen when applicate together with TAF and synergistic effects appear when coadministered with all other currently used NRTI or NVP, LPV, DRV, DTG and BIC. Earlier studies have already shown a favourable side effects profile, which didn´t show enhanced nephrotoxicity or mitochondrial toxicity as know of other NRTIs. GS-9131 is also active against HIV-2 [1,2].

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3. INTEGRASE STRAND TRANSFER INHIBITORS (INSTI)

J. Custodio discussed new data regarding the clinical pharmacology of the INSTI bictegravir (GS-9883, BIC). BIC is a new INSTI with a good oral bioavailability of >70%, and a plasma protein binding capacity of 99%. Once absorbed GS-9131 is about equally metabolized via CYP3A4 (oxidation) and UGT1A1 (glucuronidation). Neither a moderate liver nor a higher grade renal insufficiency (eGFR 15-30 mL) rise the necessity of a dose reduction in vivo. The pk-profile of BIC led to the further evaluation of BIC75mg in clinical studies, which have reached now phase 2b. The mean half-life of BIC is ~18 h, so that BIC can be taken once daily (QD). In combination with emtricitabine/tenfoviralafenamide (FTC/TAF) BIC can be dosed at 50 mg, because TAF moderately enhances the plasma concentration of BIC. Also, the influence of potent CYP3A4 inhibitors is moderate (AUC-increase of about 50%), but a concomitant induction of CYP3A4 or UGT1A1 leads to lower BIC exposure (Rifabutin: BIC AUC -38%; Rifampicin: BIC AUC -75. As BIC reaches about 20fold above the IC95 for wildtype HIV-1 at the end of the dosing interval, a dose alteration is not seen as being necessary. BIC itself is a substrate but not inhibitor or inducer of CYP3A4, so that pk-studies with midazolam, norelgestromin/ethinylestradiol, ledipasvir/sofosbuvir could not detect any drug-drug interactions (DDI). Solely the elimination of metformin via the renal transmembrane transporters OCT2 and MATE1 has been inhibited leading to a higher metformin exposure of 39%. However, since no increased incidence of side effects had been detected in these studies, a dose reduction of metformin is not suggested. Currently, BIC is further evaluated in a fixed dose combination together with FTC/TAF [3]. Recently, the US Food and Drug Administration (FDA) added to the available options for many people living with HIV with the approval of bictegravir (BIC) [4].

Paul Sax subsequently presented first data from phase 2, when BIC+FTC/TAF+Placebo (n=65) was tested vs. DTG+FTC/TAF+placebo (n=33) over 48 weeks in therapy-naive patients of a median age of 30 vs 36 years, respectively, in the majority caucasian with a mean viral load of 4.5 log10 copies/mL, a median CD4-cell count of 441 vs 455/µL and a eGFR of 130 vs 122mL/min. At weeks 24/48, 97/97% of all patients taking BIC and 94/91% of patients taking DTG were below the detection limit for HIV of 50coipies/mL; CD4-cell count increased 258 vs. 192/ µL (p=0.16). Bictegravir was also fully active against HIV-1 variants resistant to other antiretrovirals, including NRTIs, NNRTIs and/or PIs, in vitro (FC 0.8–1.9) [5,6].

Table 1. A selection of phase 2-efficacy data of BIC vs DTG, each plus FTC/TAF

N (%)	Week 24[a]		Week 48[b]	
	BIC+FTC/TAF (n=65)	**DTG+FTC/TAF (n=33)**	**BIC+FTC/TAF (n=65)**	**DTG+FTC/TAF (n=33)**
HIV-1 RNA < 50 copies/mL	63 (96.9)	31 (93.9)	63 (96.9)	30 (90.9)
HIV-1 RNA > 50 copies/mL	2 (3.1)	2 (6.1)	1 (1.5)	2 (6.1)
HIV-1 RNA ≥ 50 copies/mL	1 81.5)	1 (3.0)	0	1 (3.0)
Discontinued due to lack of efficacy	0	0	0	0
Discontinued due to other reason and at last HIV-1 RNA ≥ 50 copies/mL	1 (1.5)	1 (3.0)	1 (1.5)	1 (3.0)
No virologic data in window	0	0	1 (1.5)	1 (3.0)
Discontinued due to AE/death	0	0	1 (1.5)	0
Discontinued due to other reason and at last HIV-1 RNA < 50 copies/mL	0	0	0	1 (3.0)
Missing data in window but on drug	0	0	0	0

[a] *Difference in percentages (BIC+FTC/TAF vs DTG+FTC/TAF) at week 24: 2.9% (-8.5% to 14.2%); p=0.50*
[b] *Difference in percentages (BIC+FTC/TAF vs DTG+FTC/TAF) at week 48: 6.4% (-6.0% to 18.8%); p=0.17*

The side effects profile of both substances was comparable with focus on diarrhoea (12% in each group), nausea (8% vs 12%), headache (8% vs 3%), upper respiratory tract infections (8% vs 0%), arthralgia (6% in both groups) and back pain (6% vs. 0%). Laboratory value deviations occurred especially regarding the elevation of creatin kinase (13 vs 9%), AST-increase (9 vs 3%), hyperglycaemia (8 vs 13%), ALT-elevation (6 vs 0%), LDL-elevation (6 vs 9%), amylase-elevation (5 vs 6%), haematuria (3 vs 6%) and glycosuria (2 vs 6%). The eGFR was reduced over 48 weeks on therapy 7,0 vs 11,3mL/Min. No INSTI-resistances were detected over 48 weeks in this study.

Table 2. The effects of concomitant medication on the PK of BIC

IC Coadministered Drug(s) and Dose(S)	Dose(s) of BIC	Geometric mean ratio % (90%CI) of BIC PK with/without Coadministered Drug (n=15 for each cohort)		
		C_{max}	AUC	C_{tau}
ATV 400 mg QD	BIC 75 mg SD fed	128 (123, 134)	415 (381, 451)	NA
ATV/COBI 300/150 mg QD	BIC 75 mg SD fed	131 (123, 140)	406 (376, 438)	NA
Voriconazole 300 mg BID	BIC 75 mg SD fasted	109 (96.1, 123)	161 (141, 184)	NA
DRV/COBI 800/150 mg QD	BIC 75 mg SD fed	152 (140, 184)	174 (162, 187)	211 (195, 229)
Rifabutin 300 mg QD	BIC 75 mg SD fasted	80.4 (66.9, 96.5)	62.0 (53.2, 72.5)	44.0 (37.1, 52.1)
Rifampin 600 mg QD	BIC 75 mg SD fed	72.2 (67.7, 77.8)	24.5 (22.0, 27.3)	NA

[7]

4. NON-NUKLEOSIDALE REVERSE TRANSKRIPTASE INHIBITORS (NNRTI)

Doravirine is a new NNRTI, which could prove clinical non-inferiority in a phase 3-study when compared with a treatment consisting of darunavir/R+NRTI. These results were presented by Squires et al.t (OP45LB). Doravirine shows a distinct resistance profile, its activity remains good even when NNRTI resistance mutations such as K103N, Y181C, G190A, K103N+Y181C and E138K are detected in the HIV-1 genome. DOR can be taken QD without any food restrictions and it is evaluated currently in a fixed dose combination together with tenofovirdisoproxilfumarate (TDF) and lamivudine (3TC). In multicentre, double-blind, *double-dummy*-controlled phase 3-trial, 383 patients took either DOR+NRTI+placebo or DRV/RTV+NRTI+placebo. The median age of the mostly male (83 vs. 85%), caucasian (78 vs. 77%) patients was 34.8 vs. 35.7 years. Baseline CD4-cell counts were 433 vs. 412/µL, 87 vs. 88% took TDF/FTC and 13 vs. 13% ABC/3TC as NUC-backbone. 15 vs. 19% of all patients terminated this study prior to week 48 ab, whereas in the DOR-group one pregnancy and one death occurred other cases were due to therapeutic failure (n=12, 3%), *lost to follow-up* (n=17, 4%), withdrawal of informed consent (n=10, 3%), adverse events (n=4, 1%) or non-adherence (n=7, 2%).

The FDA-snapshot analysis stated a proportion of 84 vs. 80% of patients being below the HIV-1 detection limit of 50 copies/mL after 8 weeks on therapy 11 vs. 13% showed insufficient virologic success. The stratification of the results regarding baseline viral load (< or >100.000 and < or >500.000), baseline CD4-cell count (<50, <200, >200) or comedication (ABC/3TC or TDF/FTC) could not detect any inferiority of DOR vs. DRV.

5. PROTEASE INHIBITORS (PI)

Link and colleagues showed preclinical data of a new PI, GS-PI1, which at a very high resistance barrier in cell cultures and at comparable potency to ATV/DRV reveals a long half-life. 24h after its application the plasma concentrations in rats and dogs were several folds higher than those of the comparators ATV and DRV. Its stability in human microsomes is very high and a boosting with

COBI/RTV is not necessary. GS-PI1 could be a favourable alternative to currently used second generation PI, if further developed [4].

Table 3. Week 48 efficacy and safety outcomes

Endpoint	DOR[1] (n=383)		DRV/r[1] (n=383)		Treatment difference
HIV-1 RNA <50 copies/mL	**N**	**%**	**N**	**%**	**DOR-DRV/r (95%CI)**
Overall[2]	321	83.3	303	79.9	3.9 (-1.6, 9.4)
BL HIV-RNA ≤ 100,000[§]	285	90.2	282	88.7	1.5 (-3.7, 6.8)
BL HIV-RNA > 100,000[§]	79	81.0	72	76.4	3.0 (-11.2, 17.1)
BL HIV-RNA ≤ 500,000[§]	347	88.5	342	87.4	0.9 (-4.0, 5.9)
BL HIV-RNA > 500,000[§]	17	82.4	12	50.0	30.9 (-4.1, 65.9)
NRTI = TDF/FTC[§]	316	88.0	312	86.5	1.3 8-3.9, 6.5)
NRTI = ABC/3TC[§]	48	89.6	43	83.7	5.9 (-9.1, 20.9)
BL CD4 ≤ 200 cells/mm³[§]	41	82.9	61	72.1	9.4 (-7.4, 26.2)
Adverse Event (AE) Summary	**% of Subjects**		**% of Subjects**		**DOR-DRV/r (95% CI)**
One or more AE	80.2		78.3		1.8 (-3.9, 7.6)
Drug-related AE	30.5		32.1		-1.6 (-8.1, 5.0)
Serious AE	5.0		6.0		-1.0 (-4.4, 2.3)
Discontinued due o AE	1.6		3.1		-1.6 (-4.0, 0.6)
Fasting Lipids, change from BL	**N**	**Mean Δ**	**N**	**Mean Δ**	**DOR-DRV/r (95% CI)**
LDL cholesterol (mg(dL)	326	-4.5	318	+9.9	-14.6 (-18.2, -11.1)
Non-HDL cholesterol (mg/dL)	329	-5.3	325	+13.8	-19.3 (-23.3, 19.9)

[1]With TDF/FTC or ABC/3TC;
[2]Non-complete=Failure (NC=F) approach, as defined by FDA snapshot method; 95% CI for treatment difference based on stratum-adjusted Mantel-Haenszel method. Non-inferiority bound pre-specified as -10 percentage points; [§]Observed failure (OF) approach to missing data [5]

6. CCR5-INHIBITORS

PRO 140 is a humanized IgG4 monoclonal antibody, which can prevent the HIV entry into the target cell by binding directly to the CCR5-receptor. PRO 140 blocks genotypically diverse HIV, wildtype as well as MDR-resistant or maraviroc-resistant viruses. The CD01-study switched 16 patients from stable cART to a once-weekly application of PRO 140 monotherapy for 12 weeks. Patients who remained after 12 weeks under the detection limit of 50copies/mL (n=16) subsequently received Pro 140 for further 160 weeks (CD01-extension 2b-study). The data which were presented by Lalezari et al., revealed in this small group of patients a favourable side effects profile, good tolerability and effectivity. After 60 weeks on therapy none of the patients showed the development of Pro140 antibodies. Following more than 2 years of monotherapy, 62,5% of patients are still below DL. Further PRO140 studies have been initiated: PRO 140_CD03 investigates 300 virologic suppressed patients with CCR5trophic virus over 48weeks monotherapy. PRO 140_CD02 evaluates the application of PRO140 in 30 patients who are resistant to a number of ARVs but have CCR5-trophic virus, together with an optimized background therapy [6].

7. NANO-NNRTI AND NANO-PI

Two new *nano particle* -formulations of efavirenz (NANO-EFV) and lopinavir (NANO-LPV) were introduced by Andrew Owen. Both promised the possibility for a marked dose reduction of about 50% in population pharmacokinetic modelling studies, leading to a better tolerability especially in children, in whom both ARVs are still widely used as part of cART. When only 50% of the currently recommended dose were given the exposure ratios for EFV/smEFV (GMR AUC/C24 0.88/1.32) and LPV/smLPV (GMR AUC/Cmin 0.92/1.07) GMR, measured for Cmin und AUC, were found to be in the acceptable range of bioavailability.

Table 4. PK data of Efavirenz and Lopinavir NANO-formulations in comparison to marketed formulations

	Geometric mean		Geometric mean ratio
Efavirenz	**NANO-EFV 300 mg**	**Sustiva 600 mg**	**GMR (90% CI)***
AUC $_{0-24}$ (mg*h/L)	51.56	58.61	0.88 (0.86-0.90)
C_{12} (mg/L)	2.03	2.51	0.81 (0.78-0.83)
C_{24} (mg/L)	1.90	1.44	1.32 (1.26-1.37)
C_{max} (mg/L)	2.99	3.36	0.89 (0.87-0.91)
Lopinavir	**NANO-LPV 200 mg#**	**Kaletra 400 mg**	
C_{12} (mg/L)	4.16	4.02	1.04 (0.99-1.08)*
AUC $_{0-12}$ (mg*h/L)	72.35	79.07	0.92 (0.89-0.94)
C_{max}	10.69	9.97	1.07 (1.05-1.10)

**Nanoformulation as reference; # boosted with 100mg ritonavir [8]*

8. CAPSID INHIBITORS

The new capsid-inhibitor GS-CA1 can block steps of the HIV capsid synthesis: It inhibits not only the translocation of the preintegration complex into the genome of the target cells, but also the assembly of capsid core. The EC_{50} of GS-CA1 is only 85pM, so that GS-CA1 shows up to be much more potent than current ARVs. Although resistance mutations are known, these are exclusive for GS-CA1. Furthermore, viruses with these mutations at L56I, M66I do have e markedly reduced viral fitness. GS-CA1 shows comparable activity against multiple HIV-1 isolates of all subtypes. Capsid inhibitors do bind to a conserved binding site at the connection between to neighbouring monomers and speed up its assembly in *in vitro*.

All so far identified CAI are *in vitro* fully active against multi resistant HIV-mutants, by inhibiting the assembly of virions as well as the capsid functioning after cell entry as described before.

A single subcutaneous injection leads to long lasting high plasma concentrations, which were above the EC95 for wildtype HIV-1 longer than 10 weeks, so that GS-CA1 has the potential for a once-monthly application [9].

Table 5. A selection of new potential ARVs at CROI 2017

ARV class	Name	Structure	Studies
NRTI	GS-9131	Nucleotide-analogue	Preclinical
NNRTI	Doravirine		
	NANO-EFV	*Nano particle* Efavirenz	Bioequivalence
PI	NANO-LPV	*Nano particle* Lopinavir	Bioequivalence
INI	Bictegravir (GS-9883)	Integrase Strand Transfer Inhibitor	Phase 2/3
CCR5-inhibitor	PRO 140	IgG4 monoklonaler CCR5-Antikörper	Phase 2b
Capsid-inhibitor	GS-CA1		Preclinical/phase 1

ARV=antiretroviral; NRTI=nucleoside reverse transcriptase inhibitor; NNRTI=non- nucleoside reverse transcriptase inhibitor; PI=protease inhibitor; INI=Integrase inhibitor

9. OUTLOOK

Also, the proportion of pharmacological studies at CROI 2017 reached hardly 5%, their scientific level was nearly always high, the presented data were interesting and of clinical relevance.

New substances in clinical evaluation are scarce. Foremost, the new class of capsid inhibitors may lead to changes in future HIV therapy strategies. The new capsid inhibitor GSCA1 is reaching phase 1 now: Its very long half-life has the capacity of a once monthly subcutaneous application, so that new

substance with comparably pk-profiles may lead to substantial changes in future therapy regimens: Uncomplicated application, very long dosing intervals, low/no pill burden, improved adherence.

Also, the new nanomolecular formulations of already widely used substances with the potential of decrease dosing and improved tolerability, may help to improve HIV treatment especially in children. Once more, this shows the need and benefit of university-based pharmacological science, since the further improvement of both substance is of no economic interest for manufacturers.

10. CONCLUSION

There are a number of promising candidates for antiretroviral treatment in the development and approval pipeline. Bictegravir and Doravirine have already been approved in 2019/2020 and were introduced to the market either as new single-tablet formulation (Bictarvy ®, together with TAF) or single drug (Pifeltro ®, to be combined with two NRTI). These new antiretrovirals complement the currently available treatment options.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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Study on Potential Drug Interactions between cART and New Psychoactive Substances

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ABSTRACT

New hazardous drugs with unknown synthetic chemical structures or structures of known entities that have been changed in order to avoid prosecution are flooding the European illegal drug market. Tons of synthetic cannabinoids, synthetic phenylethylamine/cathinones or substances of rather rare occurrence have been seized by authorities during the past years, which is believed to only the tip of the iceberg. These substances are marketed as *legal highs,* often legally sold via internet structures and especially consumed by very young people as party drugs. Their mechanisms of action, hazardous side effect profile and potential drug-drug-interactions, especially with antiretrovirals, are discussed in the following article.

Keywords: Legal highs; synthetic illegal drugs; drug-abuse; drug-drug-interactions.

1. INTRODUCTION

So-called *designer drugs* are drugs with so far unknown synthetic chemical structures or structures of known entities that have been changed in order to avoid prosecution. In the past years, a number of these substances emerged on the European (and US) drug market: 164 new psychoactive substances have been reported by the European rapid alert system of the EMCDDA (European Monitoring Center for Drugs and Drug Addiction) between 2005 and 2014. Synthetic cannabinoids and synthetic phenylethylamine/cathinones are responsible for about two thirds of all new substances but also a number of substances of rather rare occurrence were seized. Often, the molecular structure of common narcotics is changed, so that law prosecution doesn´t take effect, but drug effects remain or are even increased. These drugs are marketed as *legal highs, research chemicals, herbal mixtures, air refreshers, bath salt* or even *plant fertilizer* without showing the ingredients or chemical structures. The manufacturers give the impression that these drugs are safe and nonhazardous.

Table 1. New psychoactive substances, of which the first six are not underlying law enforcement until at least 2014

Substanz (engl.)	Jahr	Name
1-cyclohexyl-4-(1, 2-diphenylethyl) piperazine	2014	MT-45
4-methyl-5-(4-methylphenyl)-4, 5-dihydrooxazol-2-amine (4, 4′-dimethylaminorex, 4, 4′-DMAR)	2014	4,4´-DMAR
1-(1, 3-benzodioxol-5-yl)-2-(pyrrolidin-1-yl)pentan-1-one	2014	MDPV
2-(3-methoxyphenyl)-2-(ethylamino)cyclohexanone	2014	Methoxetamin
2-(4-iodo-2, 5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine	2014	25I-NBOMe
3, 4-dichloro-N-{[1-(dimethylamino)cyclohexyl]methyl}benzamide	2014	AH-7921
N,α-dimethylbenzeneethanamine (alt: 4-methylamphetamine)	2014	Methamphetamin
4-methylmethcathinon	2011	Mephedron

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In contrast to that, these drugs can have harmful side effects such as circulatory collapse, syncope, paralysis, psychosis or even death [1].

Besides the already well-known metamphetamines and mephedrone, in particular six new substances have gained in importance during the past 10 years. Most of these have not been subject to law enforcement until at least 2014 (see Table 1).

2. THE NEW "LEGAL-HIGHS"

Whereas ketamine, GHB or metamphetamine are probably well known by the majority of HIV-therapists, it may be worthwile focusing on drugs that have reached the “market” during the past two years: MT-45, 4,4´-DMAR, MDPV, Methoxetamin, 25I-NBOMe und AH-7921 [1,2].

2.1 MT-45

MT-45 (alias IC-6) is typically applicated orally or via nasal insufflation, the gas of the heated free base can be inhaled, the water-soluble hydrochloride-salt can be injected i.v. One single dose amounts between 15-30mg (nasal) and 25-75mg (oral). Users often take more than one dose consecutively. Effects occur 15 (nasal) to 60 (oral) minutes after intake and last about 2 hours. The effects correspond to other opioids and estimates a magnitude of ~0.8 in correlation to morphine. MT-45 causes no euphoria but sedation and a potent analgesic effect is stated by consumers. (see Drugs Forum (https://drugs-forum.com/forum), a relevant website, where users report their experiences with new substances) MT-45 is a selective μ-opioid receptor (MOR-1) agonist showing nM binding affinities to MOR-1, but is less potent than morphine, with considerably lower *δ*- and *κ*-opioid receptor (DOR-1 and KOR-1) affinities [3,4].

There are no data existing about the pharmacokinetics of MT-45 and so far, no metabolites of the degradation in human organisms have been identified. The racemic formulation of MT-45 has the same opioid effects as the single (S)- and (R)-enantiomeres, whereat the (S)-enantiomer is several times more potent than morphine. MT-45 works as an agonist of the κ- and δ-opioid-receptors with much higher affinity than morphine itself. The acute adverse effects are obvious and are especially constipation and respiratory depression. The acute toxicity of MT-45 is much higher in correlation to morphine, especially after intravenous injection [5,6].

2.2 4,4´-DMAR

Regularly, 4,4´-DMAR (street name is chemically incorrect “Aminorex”) has been detected as addition to ecstasy tablets or as blend of cocaine sold in the street. The application route is usually nasal or oral and a typical dose amounts to 10-60 mg, single cases reported of the intake of up to 200 mg [7]. 4,4′-DMAR was identified for the first time in 2012 in seized white powder at a national focal point in the Netherlands [8] and reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) [9,10].

There are no data existing about the pharmacokinetics of 4,4´-DMAR and so far, no metabolites of the degradation in human organisms have been identified.

Up to date, the pharmacology has only been evaluated in in-vitro-studies and could show that 4,4´-DMAR even in very low concentrations in the CNS increases the release of dopamine (DA, EC50 8.6), norepinephrine (NE, EC50 26.9 nM) und serotonin (5-HT, EC50 18.5 nM) [11].

Although studies in humans are lacking, 4,4`-DMAR most probably interacts pharmacodynamically with SSRI, MDMA or cocaine. Especially, it indicates the danger of developing a serotonin-syndrome. High doses and/or the concomitant application with other catecholamine-releasing drugs in the CNS lead to psychotic symptoms, agitation and potentially hazardous cardiovascular events via norepinephrine-release in the periphery. Consumers report about euphoria, mental and physical stimulation, empathic effects or changes of visual perception. In 2014, 31 fatal casualties were

registered throughout Europe, especially caused by hyperthermia, convulsions, respiratory problems and cardiac arrest [12-14].

2.3 MDPV

MDPV (street names *Flakka, Flex, Cloud Nine, Monkey Dust, MTV, Magic, Super Coke, Peevee*) is one of more than 50 cathinones, which have been reported by the EMCDDA in the past years; other examples are mephedrone (4-MMC) and methylone (bk-MDMA). MDPV is marketed typically as bath salt, which can be solved in water and injected i.v. or subcutaneously. There also exist tablet- and capsule-formulations and sometimes rectal application or nasal insufflation. Common doses are 5-11mg (insufflation), 8-15mg (oral) and 6-12mg (rectal). The wanted effects occur about 30minutes after intake and last 2 to 7 hours.

MDPV inhibits selectively the catecholamine uptake (via dopamine transporter, DAT, or norepinephrine transporter, NET), while serotonin uptake is less affected [15]. In animal models, the effects of MDPV lasted much longer than those of cocaine [16,17].

Phase-1 metabolism, which was found in animal and in-vitro studies include demthylation, followed by methylation, aromatic side chain-hydroxylation and oxidation of the pyrrolidine to its corresponding lactam and the opening of the cycle to a carboxyl acid [18-21]. No data exist about the biological activity of these metabolites.

When seized, MDPV was found often blended with lidocaine, procaine, piracetam, trimethoprim or diltiazem as well as cocaine, ketamine, methamphetamine, TFMPP, BZP, mephedrone, methylone, 4-MEC, MDPBP, alpha-PVP and synthetic cannabinoids. Results of various *in-vitro*- and *in-vivo*-animal studies show an effect on behavior comparable to cocaine or methamphetamine.

The pharmacological key mechanism is the inhibiton of catecholamine transporters as mentioned above, whereas MDPV is 10-50 times more potent in this regard than cocaine. MDPV e.g. increased the dopamine concentration in CNS structures of rats significantly, leading to hyperthermia and hypertonia.

The hepatic metabolism of MDPV involves the cytochrome oxidases (CYP) 2C19, 2D6 and 1A2 [21], which has importance regarding genetic polymorphisms in the expression of the cytochromes in humans as well as potential DDIs (shown in Table 2).

The typical effects are comparable to those of cocaine, amphetamines and mephedrone and are shown in Table 2 [22]. There also have been reports about malignant hyperthermia, rhabdomyolysis, renal failure and stroke [23-26]. 108 fatal casualties have been reported in the EU until 2014 in relation to the use of MDPV [27].

2.4 Methoxetamin

Regarding its structure, methoxetamine is comparable to ketamine [28-30]. Usually methoxetamine is applicated via nasal insufflation, orally taken but can also be solved in water and injected i.m. or i.v.

An *in-vitro* study could show that methoxetamine like ketamine is affine to the NMDA-(*N*-methyl-D-aspartate)-receptor [31], but other than ketamine also to the serotonin transporter [32].

It seems by descriptions of users, that effects are comparable to those of ketamine, a dissociative analgesic drug and are euphoria, increased empathy, intense sensory effects, dissociation from the physical body, derealisation, feelings of security, increased social interaction, hallucinations, introspection and short-time antidepressive effects [33]. Methoxetamin is taken in single doses of 10-200 mg, although a single dose should not extend 50 mg. The effects occur about 30-90 minutes after nasal insufflation, 90 minutes after oral intake and 5 minutes after i.v. injection and last about 1-7 hours.

Two studies evaluated the pharmacokinetics of methoxetamine and found a number of phase-I and phase-II metabolites, which evolved from demethylation, oxidation and glucuronidation [32,34].

The toxic profile of methoxetamine resembles largely to ketamine. Methoxetamine was seized either alone or in blends together with other stimulants like caffeine, lidocaine or phenacetine.

Up to 2014 about 120 non-fatal intoxications and 20 fatal casualties have been registered throughout the EU. Toxic effects are shown in Table 2. An acute intoxication often shows up with agitation, tachycardia and hypertensive crisis, which is similar to ketamine, but also unlike ketamin with ataxia and nystagmus [35].

So far, no data exist about potential pharmacokinetic DDIs with other drugs, but pharmacodynamic interactions can occur with other CNS-effective substances such as alcohol, in this case aggravating depressive and respiratory depression effects. Since the concomitant intake of diazepam and other benzodiazepines reduces the plasma clearance of ketamine, it may be suggested that this could also be possible with methoxetamine.

Regarding the cART, higher doses of methoxetamine may interfere with integrase inhibitors dolutegravir/raltegravir, which are also glucuronidated via UGT, but this has not been proven yet and may only be concluded from so far known metabolic pathways of these drugs.

One publication from 2015 shows also a role of CYP 2B6 and 3A4 regarding the hepatic metabolism of methoxetamine, leading to potential DDIs with ritonavir and/or cobicistat. However, this is unclear and potential effects of generated metabolites remain unknown [32].

2.5 25I-NBOMe

25I-NBOMe (street name: 2C-I-NBOMEe, 25I-N-Bomb) had been produced in the early 2000s. Typically, 25I-NBOMe is seized as paper-trips, i.e. absorbent paper, saturated with 25I-NBOMe for a sublingual application. The typical dose ranges from 500 to 800µg and its effects occur after 15-120min and last about 6-10 hours.

A number of pharmacological in-vitro and animal studies have evaluated the PK and PD of 25I-NBOMe. It was proven in cell-assays that 25I-NBOMe is a full 5-HT2A-receptor-agonist with a high receptor-affinity even at very low nanomolar concentrations. The addition of a N-(2-methoxybenzyl) group increased markedly the receptor binding capacity in comparison to e.g. 2C-I.

Reports of users suggest that 25I-NBOMe has foremost hallucinogenic effects. The results of animal studies, which used the *head, twitch behavioural response* (HTR) e.g. in mice as marker for hallucinogenic effects of a 5-HT2A-receptor activation, confirmed these statements.

25I-NBOMe produced a distinct and lasting effect in the HTR, which could be reversed by the 5HT2A-receptorantagonist volinanserin. However, 25I-NBOMe was about 10-fold more potent than 2C-I in these test arrangements. Furthermore, affinity to other receptors in the CNs, e.g. Weitere 5-HT1A/2B/2C, 5-HT6, dopamine D3 and D4, α2C adrenoreceptor and serotonin transporter (SERT), was found.

25I-NBOMe is mainly metabolized by O-demethylation, O,O-bis-demethylation, hydroxylation, and combinations of these reactions as well as subsequent glucuronidation and sulfatation (phase II). All together, 68 metabolites have been identified. A screening of cytochromoxidases showed that also CYP 1A2, CYP 3A4, CYP 2C9/2C19 are involved in metabolism [36]. The last involved CYP2C9/2C19 lead to O-demethylation, and the emerging metabolite N-(2-Hydroxybenzyl) is also a strong 5HT receptor-agonist. Further metabolites have not been identified [37].

Effects include those of other psychoactive substances (e.g. LSD, psilocybin or 2C-B (two, 5-dimethoxy-4-bromo-phenethylamine)), but clinical case reports have also shown serotoninerge intoxication such as agitation and confusion. Some users described severe psychic behavioural deviations with the use of 25I-NBOMe: These include intense auditive and visual hallucinations, severe agitation, aggression and unpredictable outbreaks of violence, which -in some cases-led to trauma or death (effects and side effects are shown in Table 2).

Interactions with other medication o drugs are difficult to predict, but pharmacodynamically it is most probable that additive toxicities occur when concomitantly taken with other serotoninerge substances, e.g. SSRI, which increase serotonin concentrations in the CNS. Symptoms of a serotoninerge syndrome are tachycardia, hyperthermia, hypertonia, muscle rigidity and convulsions. Possible pharmacokinetic interactions are listed in Table 2.

Information of users and from seizures proved that 25I-NBOMe is sold as “legal” substitute for LSD or is sometimes directly marketed as LSD on the illegal market [37].

2.6 AH-7921

AH-7921 (street name “Doxylam”) structurally is an atypical synthetic morphine opioid analgesic, which has already been found in the mid 70ies of the last century. Chemically it is a derivate of hylaminocyclohexan [38]. As a synonym for AH-7921 is “doxylam” it is sometimes mixed up with doxylamin, an internationally available generic of an antihistaminic drug with also hypnotic and sedating effects. For this reason, doxylam could be taken as doxylamin with a high danger of overdosing, since both drugs have a distinct dosing range.

Various studies in animals and in vitro-evaluations described the pharmacodynamic characteristics, e.g. sedation and analgesia of AH-7921: AH-7921is a morphine-like analgesic, with a main effect as μ-opioid (MOP)-receptor agonist and a lower affinity tot he κ-opioid (KOP)-receptor. Antagonists are e.g. naloxone, which is atypical opioid-antagonist used in clinical settings. A number of animal studies proved a high analgesic potency of AH-7921, being several-fold that of codeine and comparable to that of morphine. It has also been found that analgesic doses were close to toxic doses, so that it has a very narrow therapeutic window. The side effects profile is comparable to that of morphine, but it´s respiratory depressive potency is 1.6-fold higher.

Data of post-mortem investigations in humans showed the formation of two *N*-desmethyl metabolites, which are products of a sequential *N*-demethylation of the *N,N*-dimethylamino groups of the original compound. So far, there is also no information about a biological activity of these metabolites existing. The lethal dose (LD50) of AH-7921 in mice is 10 mg/kg when intravenously injected [39]. Further side-effects-studies in animals could define a steep linear dose-effects relation with a high risk for respiratory depression, comparable to that of morphine. Commonly taken doses range between 40 and 150mg, often enhanced by a subsequent intake of further doses in order to extend the effects up to 6-12 hours. The potential for developing addiction is more or less that of classic morphine [40]. Until 2014, 15 deaths related to the intake of the drug have been reported in the EU.

As the pk of AH-7921 has not been fully evaluated, no data about interactions with other drugs or medical compounds are available [41]. However, sedating effects are most probably comparable to morphine [42]. Meanwhile AH-7921 has been prosecuted in some countries worldwide, e.g. the UK (January 2015), Brasil (May 2015) or China (October 2015).

3. OUTLOOK

Finally, this overview underlines the lack of knowledge about the pharmacology of the new, so-called *legal highs,* synthetic drugs, which in many cases are not prosecuted but sold legally e.g. via the internet. Systematic studies of pharmacokinetic interactions with cART or other medical products are lacking, so that therapists can only warn patients about an incalculable risk of taking these drugs together with ARVs. Therapists must understand that, if severe adverse events or failing cART are reported in this context, documentation and evaluation of these cases can add to the knowledge of the public. In this context, it is crucial that patients are willing to report the consumption of and experience with *legal highs* in a confiding relationship to their therapists. Therapists have to ask about a possible consumption of these drugs and should update regularly their knowledge about these drugs in order to be able to assess possible correlations to a failure of cART or DDIs.

The ignorance about the fast growing use of *legal highs* is a crucial threat to an effective prevention and therapy of HIV all over Europe.

As discussed above, exact medical and pharmacological information can be accessed via the internet websites of the EMCDDA [43] and other internet based sources, specialized on this issue.

Table 2. Overview of effects, pharmacology and potential DDIs of the new designer drugs

Substances	Uptake	Pk/Metabolism	Pharmakodynamics	Effects	Toxicology	Interactions (PD)	Interactions (PK)
MT-45	nasal, oral, inhalation, i.v.	unknown	κ- and δ-opioid-agonist	euphoria, dissociation	respiratory depression, obstipation	unknown	unknwon
4,4´-DMAR („Aminorex“)	Nasal, oral, i.v.	unknown	dopamine-, serotonin und epinephrin-release	euphoria, stimulation, altered visual perception	agitation, hyperthermia, psychosis, convulsions, cardiac arrest	possible with efavirenz* SSRI, MDMA, cocaine	unknown
MDPV	Nasal, oral, rektal, s.c., i.v.	CYP 2C19 CYP 2D6 CYP 1A2	dopamien- and epinephrin-reuptake-inhibiton	Euphoria, stimulation, altered visual and auditive perception	e.g. tachykardia, convulsions, respiratory problems, depression, confusion, agitation, anxiety, auditive and visuelle hallucinations, paranoid psychosis	nelfinavir, fluoxetine, carbamazepine, moclobemide, tramadol, haloperidol, diltiazem, citalopram, caffeine, diazepam, cannabis, olanzapine, warfarine	buproprione, paroxetine, fluoxetine, quinidine, duloxetine, sertralin, terbinafine [1] possible with ritonavir
Methoxetamine	Nasal, oral, rektal, i.m., i.v.	demethylation oxidation glucuronidation CYP 2B6 CYP3A4	NMDA-receptor-agonist SERT-inhibiton	euphoria, empathy, intense sensoric effects, dissociation, derealisation, increased social intercourse, hallucinations,intros pection and antidepressive effects	Vomiting, diarrhea, bradycardial arrhythmia, loss of conscience, sweating, sight disorders, headache, tremor, disorientation, depression, fear, katatonia, confusion, agiation, aggression, hallucinations, paranoia, psychosis	diazepam, alcohol	raltegravir, dolutegravir ritonavir, cobicistat, atazanavir, efavirenz

Substances	**Uptake**	**Pk/Metabolism**	**Pharmakodynamics**	**Effects**	**Toxicology**	**Interactions (PD)**	**Interactions (PK)**
25I-NBOMe	Nasal, (oral), sublingual, inhalativ, rectal, i.m., i.v.	CYP1A2, CYP3A4, CYP2C9 CYP2C19	5HT2A-agonist (main effect) 5HT-agonist SERT-inhibiton	auditive and visual hallucinations	Serotoninergic syndrome tachcardia, hyperthermia, hypertonia, convulsions, muscular rigidity, agitation, aggression, burst of violence	SSRI, and other serotoninergic substances	possible with cART **
AH-7921	Oral, i.v.	N- demethylation (CYP??)	μ-opioid -receptor-agonist minor affinity to κ-opioid –receptor	analgesic effects	Respiratory depression, obstipation, vomiting, nausea	hypnotics, anxiolytics, tricyklic antidepressants sedating antihistamnics	unknown

PK = pharmacokinetic interactions; PD = pharmacodynamic interactions
**No study data, metabolism via cytochrome oxidases is suspected or **has been proven*
[1] http://medicine.iupui.edu/clinpharm/ddis/main-table/

4. CONCLUSION

So-called *legal highs*, a number of diverse synthetic drugs, which are flooding the European illegal drug market during the past years are not only a threat to the health of their consumers but also to the efficacy of an antiretroviral therapy. Their potency for drug-drug-interactions is high and often unknown by prescribers and users of ART. More studies on these drugs and more information for doctors and patients are warranted in order to avoid hazardous results after consumption of *legal highs* together with ART.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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Recent Advancements of High-Performance Thin Layer Chromatography Method for Identification and Quantification of Oleanolic Acid in the Roots of *Achyranthes aspera* Linn.

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ABSTRACT

Aim: A correlation exists between biological activities and presence of secondary metabolites in plants. The present research work is carried out to develop and validate a chromatographic method using HPTLC for identification and quantification of Oleanolic Acid from the roots of *Achyranthes aspera* Linn.
Methods: The External Standard Method is used for quantification analysis in present study. It assures accuracy and precision in quantitative analysis and is especially suited for HPTLC studies. Chromatography was performed on aluminium-backed silica gel $60F_{254}$ HPTLC plates of 250 µm thickness with solvents toluene: ethyl acetate: Methanol: Acetone 14:4:1:1 (v/v/v/v) as the mobile phase. Derivatisation was carried out with Anisaldehyde sulphuric acid, scanned, and quantified at 540 nm.
Results: The concentration of Oleanolic Acid in roots of the plant is found to be 1.277ng/ µg. The statistical analysis proved that the developed method is suitable and specific.
Conclusion: The developed HPTLC technique can be used for the routine quality control analysis and quantitative determination of oleanolic acid from *A. aspera* Linn. The oleanolic acid was found to be linear in the range of 0.20-0.60 µg/µl. Considering the wide therapeutic applications of oleanolic acid an alternative quantification technique of this marker constituent was generated to ensure identity and quality of the selected plant. This is a sensitive, specific and reproducible HPTLC method for the quantification of Oleanolic Acid from roots of *Achyranthes aspera* Linn.

Keywords: Achyranthes aspera Linn.; quantification; oleanolic acid.

1. INTRODUCTION

Herbal medicines have a strong traditional or conceptual base and the potential to be useful as drugs in terms of safety and effectiveness in treating different diseases. The use of herbal medicines and phytonutrients or nutraceuticals continues to expand rapidly across the world with many people now resorting to these products for treatment of various health challenges in different national healthcare settings [1,2]. The World Health Organization has made an attempt to identify all medicinal plants used globally and listed more than 20,000 species. A correlation exists between biological activities and the presence of secondary metabolites in plants. Many secondary metabolites have been isolated from plants, and their pharmacological activity has been established. It is estimated that up to four billion people (representing 80% of the world's population) living in the developing world rely on herbal medicinal products as a primary source of healthcare and traditional medical practice which involves the use of herbs is viewed as an integral part of the culture in those communities [3,4]. Oleanolic acid is one such secondary metabolite, with established medicinal properties, reported from medicinally important plant *Achyranthes aspera* Linn.

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A. aspera Linn. is found on road sides, field boundaries, and waste places as a weed throughout India up to an altitude of 2100 m and in the South Andaman Islands [5,6]. Although it has many medicinal properties, it is particularly used spermicidal, [7] antipyretic [8] and as a cardiovascular agent [9] *A. aspera* Linn. is a well-known plant drug in Ayurvedic, Unani-Tibbi, Siddha, Allopathic, Homeopathic, Naturopathic & Home Remedies [10,11]. It is used by traditional healers for the treatment of fever, dysentery, and diabetes [12]. Traditionally for snake bites, the ground root of *A. aspera* Linn. is given with water until the patient vomits and regains consciousness [13]. Fresh piece of the root of is used as tooth brush. A paste of the roots in water is used in ophthalmic and opacities of the cornea [14]. Roots are used as astringents to wounds, in abdominal tumor and stomach pain [15].

Oleanolic acid is found in roots of *A. aspera* Linn. and can be used as biomarker to establish the authenticity of the plant. Oleanolic acid (3β-hydroxyolean-12-en-28-oic acid) is a pentacyclic triterpenoid compound possessing broad range of biological activities. Oleanolic acid and its derivatives possess several promising pharmacological activities, such as hepatoprotective effects, and anti-inflammatory, antioxidant, or anticancer activities [16]. Oleanolic acid is reported in *A. aspera* Linn. and present study is aimed at identification and quantification of oleanolic acid from roots of the plant under study. Such natural products can be employed as biomarkers and effectively used for the purpose of authentication of plant material [Fig. 1].

1.1 Oleanolic Acid

Synonyms: (3β)-3-hydroxyolean-12-en-28-oic acid; (+)-oleanolic acid; 3β-Hydroxyolean-12-en-28-oic acid; astrantiagenin C; caryophyllin; giganteumgenin C; gledigenin 1; NSC 114945; oleonolic acid; virgaureagenin B;

Molecular formula: $C_{30}H_{48}O_3$

Molecular weight: 456.70

Chemical class/group: Terpenes (Subclass: Triterpenes, pentacyclic trirerpenes)

2. MATERIALS AND METHODS

In the present research work, an attempt has been made to develop a high-performance thin layer chromatography (HPTLC) method for quantitative determination of oleanolic acid from root powder of *A. aspera* Linn. The External Standard Method is generally used for quantification analysis in TLC studies as assures accuracy and precision in the quantitative analysis. A chromatogram was developed using standard oleanolic acid with different concentration and root extract with same concentration, plotted separately on HPTLC plate. A calibration curve was obtained by plotting standard peak area against concentration as per the ICH guidelines [17,18].

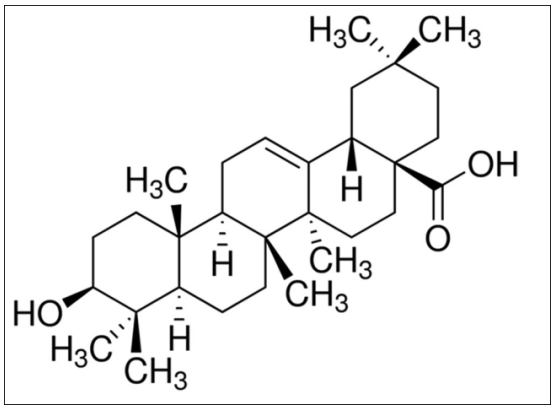


Fig. 1. Structure of oleanolic acid

Methanolic extracts of root powder was used for the experimental work. Separation was performed on an aluminum-backed silica gel $60F_{254}$ HPTLC plates, with 250 µm thickness; E. Merck, Darmstadt, Germany, using toluene: ethyl acetate: methanol: acetone in the volume ratio of 14:4:1:1 (v/v/v/v) as mobile phase for oleanolic acid. After development, the plate was derivatized with anisaldehyde sulfuric acid reagent. Detection and quantification were performed by densitometry at λ = 540 nm in visible mode. Evaluation was performed by comparing peak areas with linear regression.

Oleanolic acid was not detectable in UV light or fluorescence therefore it has been transformed in detectable substances to evaluate the TLC separation. Derivatization of the HPTLC plate was done by dipping the plate in anisaldehyde sulfuric acid reagent for <5 s. After removing the plate from the solution, it was air dried first and then CAMAG plate heater II, at 110-120°C for 4-5 min. The plate was scanned at 540 nm using tungsten lamp by CAMAG Scanner IV and Wincat software version 1.4.6 for *β*-sitosterol. The chromatographic conditions are given in Table 1.

2.1 Instrument

Camag Linomat V sample applicator, Camag Twin trough glass chamber and Camag TLC Scanner IV equipped with Cats 1.4.6 version software.

Table 1. Optimized chromatographic conditions for identification and quantification of oleanolic acid in roots of *A. aspera* Linn

Parameters	Description
Stationary Phase	Silica gel 60 F_{254} pre-coated on aluminium sheet
Mobile Phase for Oleanolic Acid	Toluene:ethyl acetate:methanol:acetone in the volume ratio of 14:4:1:1 (v/v/v/v)
Prewashing of the plate	Methanol and activated at 110°C for 30 min.
Chromatographic chamber	CAMAG Twin Trough Chamber
Chamber Saturation time	20 min
Sample applicator	CAMAG Linomat V
Band length	8 mm
Development distance	80 mm
Derivatizing reagent	Anisaldehyde sulphuric Acid
Drying of plate	At 110°C for 5 min
Densitometric scanner	CAMAG TLC scanner IV
Lamp	Tungsten
Wavelength	540 nm
Chromatographic evaluation	CAMAG TLC software Wincats 1.4.6

A. aspera: Achyranthes aspera

2.2 Reagents

Toluene, Ethyl acetate, methanol and acetone were of analytical reagent grade with 99.8% purity. They were obtained from SD Fine chemicals.

2.3 Standards

Standard oleanolic acid was procured from Sigma-Aldrich.

2.4 Glassware

Standard volumetric flasks and pipettes of class A grade were used throughout the determination.

2.5 Plant Material

Whole plants of *A. aspera* Linn. were collected in the month of August and September 2013 from natural habitats in Vasai region of Thane district. The plants were authenticated at Blatter's herbarium;

St. Xavier's College, Mumbai and the specimens voucher were deposited in the St. Xavier's College Herbarium for further reference. The accession number for *A. aspera* L. is 62490.

2.6 Preparation of Standard Solutions for Oleanolic Acid

2.6.1 Preparation of stock (A) solution of oleanolic acid (1 µg/µl)

Stock (A) solutions of oleanolic acid (1 µg/µl) were prepared in methanol. 10.0 mg of standard oleanolic acid was accurately weighed and transferred to a 10.0 ml standard volumetric flask. The contents of the flask were initially dissolved in 5.0 ml of methanol, followed by sonication and then diluted up to the mark with methanol.

2.6.2 Preparation of stock (B) solution for oleanolic acid (0.1 µg/µl)

From the standard stock (A) solution, 0.1 ml is transferred to a 10.0 ml standard volumetric flask. The contents of the flask were initially dissolved in 5.0 ml of methanol, followed by sonication and then diluted up to the mark with methanol. Thus, a working stock solution of oleanolic acid of 0.1 µg/µl was prepared in methanol.

2.7 Preparation of Mobile Phase

The mobile phase comprising toluene: ethyl acetate:methanol:acetone in the volume ratio of 14:4:1:1 (v/v/v/v) was prepared. Twin trough chromatographic chamber was saturated for 20 min with Whatmann paper No.1.

2.8 Preparation of Sample Solution

Oleanolic acid is soluble in methanol, and hence methanol was used for extraction from plant powder during method development and validation for the plant. Root extracts of the concentration 50 µg/µl were prepared. During the process, 500 mg of root powder of *A. aspera* Linn. was extracted with 10.0 ml of methanol. The mixture was sonicated for 30 min and it was kept overnight for extraction. It was filtered through Whatmann filter paper No. 41 and filtrate obtained was subjected to HPTLC for quantification of oleanolic acid. 10 µl of the sample solution was applied along with standard solution for quantification.

2.9 Preparation of Chromatogram

The quantification studies were carried out in accordance to External Standard Method by applying different concentrations of standard oleanolic acid (0.1 µg/µl) and same concentration of sample solution (50 µg/µl) on HPTLC plate [Table 2].

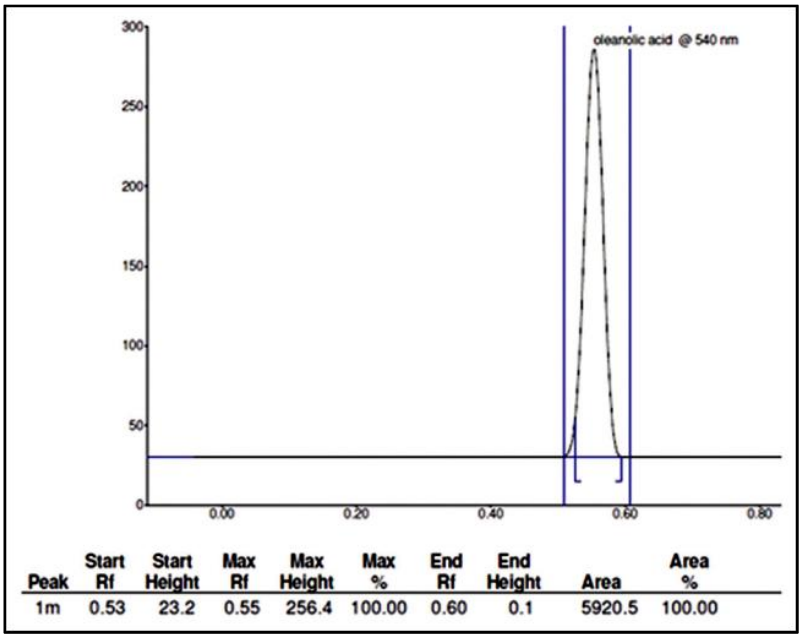
3. RESULTS AND DISCUSSION

3.1 Identification

The identity of the band of oleanolic acid in root extract was confirmed by comparing R_f value of root extracts with that of standard solutions [Figs. 2 and 3].

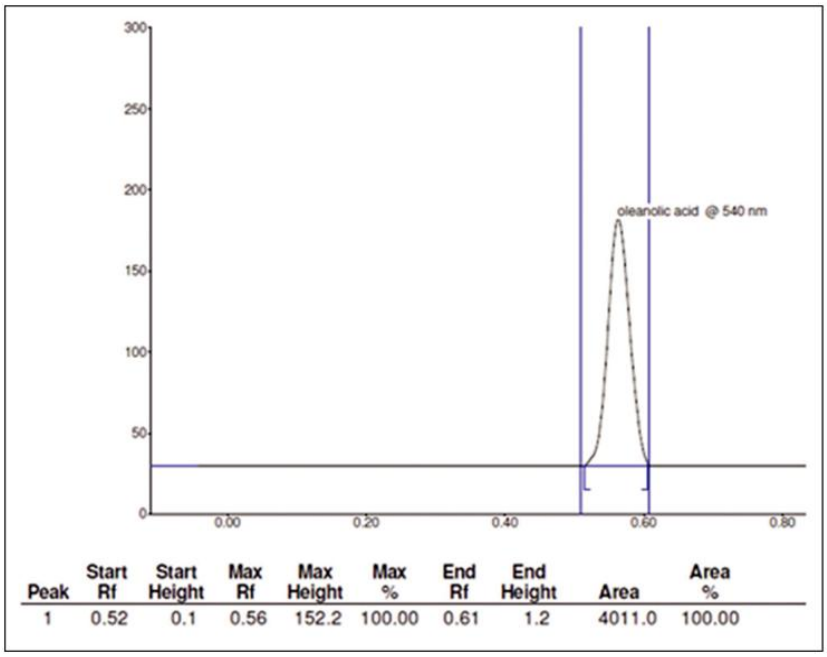
3.2 Quantification

The HPTLC densitogram [Fig. 5] and HPTLC profile [Plate 1] are obtained using standard HPTLC procedure [Table 1]. Chromatogram of standard oleanolic acid solution with volume ranging from 4 µl to 2 µl yielded better results and hence were used for the analysis. Similarly, 4 readings of standard root extract were used for the purpose of quantification as per the guidelines.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1m	0.53	23.2	0.55	256.4	100.00	0.60	0.1	5920.5	100.00

Fig. 2. Chromatogram of Oleanolic Acid with applied vol. 5 µl



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.52	0.1	0.56	152.2	100.00	0.61	1.2	4011.0	100.00

Fig. 3. Chromatogram of of root extract of *Achyranthes aspera* Linn

Graph of peak area and concentration of oleanolic acid in root extract of *A. aspera* Linn. when plotted, shows linear relationship. The linear regression equation is obtained from this graph [Fig. 4]. Using the regression equation of the linear regression graph, the amount of standard oleanolic acid applied on plate is calculated [Table 2]. Similarly, the amount of oleanolic acid a 10.0 µl root extract Sample was calculated and the results are given in Table 3. The concentration of oleanolic acid in the root of the plant is found to be 1.279 ng/µg.

Table 2. The Rf values and peak areas corresponding to the serial dilutions of standard compound-oleanolic acid and fixed amount of root extract of *A. aspera* Linn.

Track No.	Applicator sample	Applicator volume (µl)	Amount per spot (µg)	Rf	Peak area
1	Oleanolic Acid	6.0	0.6	0.55	6816.70
2	Oleanolic Acid	5.0	0.5	0.55	5920.54
3	Oleanolic Acid	4.0	0.4	0.56	4923.12
4	Oleanolic Acid	3.0	0.3	0.56	3790.93
5	Oleanolic Acid	2.0	0.2	0.56	2655.74
8	Root extract	5.0	250	0.56	4011.00
9	Root extract	5.0	250	0.57	3971.71
10	Root extract	5.0	250	0.57	3961.49

A. aspera: Achyranthes aspera

Table 3. The amount of oleanolic acid per 5.0 µl of different root extracts of *A. aspera*

Serial number	Applicator sample	Applicator volume (µl)	Amount per spot (µg)	Rf	Peak area	Amount of Oleanolic Acid (ng)
1	Root extract	5.0	250	0.56	4011.00	322.51
2	Root extract	5.0	250	0.57	3971.71	318.70
3	Root extract	5.0	250	0.57	3961.49	317.72
Mean						319.64
SD						2.53
%CV/RSD						0.792

A. aspera: Achyranthes aspera, CV: Coefficient of variation, RSD: Relative standard deviation

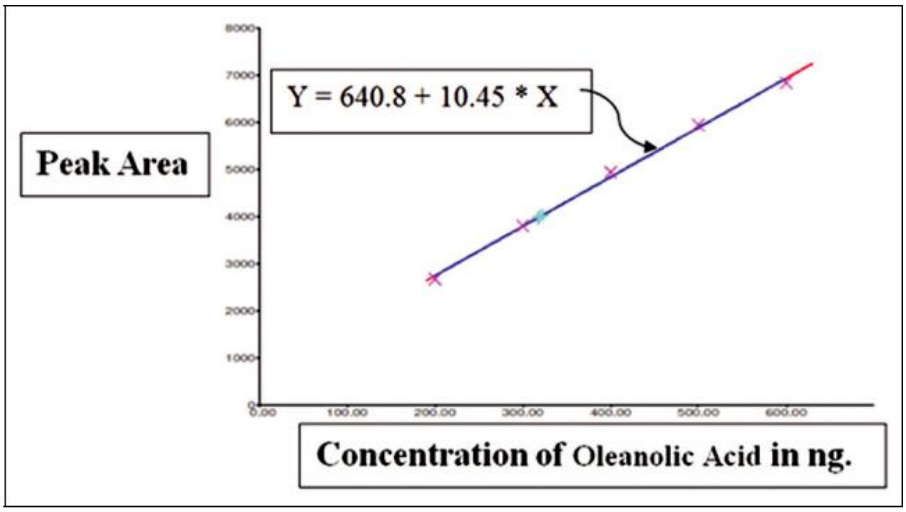


Fig. 4. Graph of peak area and concentration of Oleanolic Acid and root extract of *Achyranthes aspera* Linn

Distributions with a coefficient of variation (% CV) in the above results obtained is <1 indicating low-variance and thus it can be claimed that the results are fairly reliable.

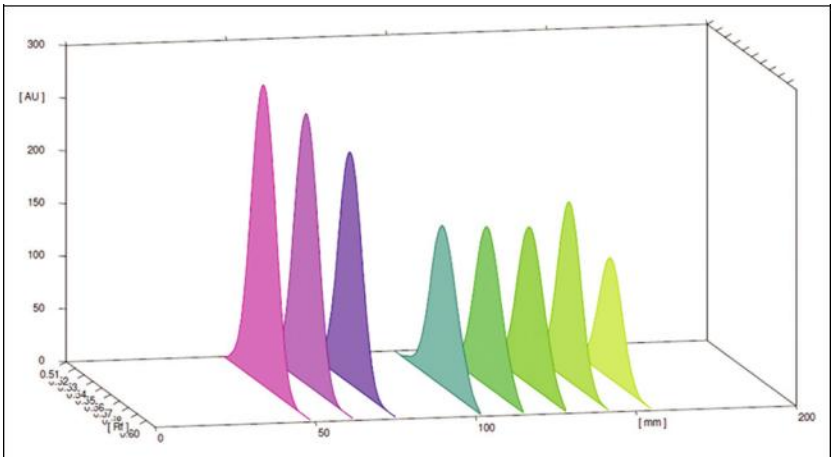


Fig. 5. Densitograms of Oleanolic Acid and root extract of *Achyranthes aspera* Linn

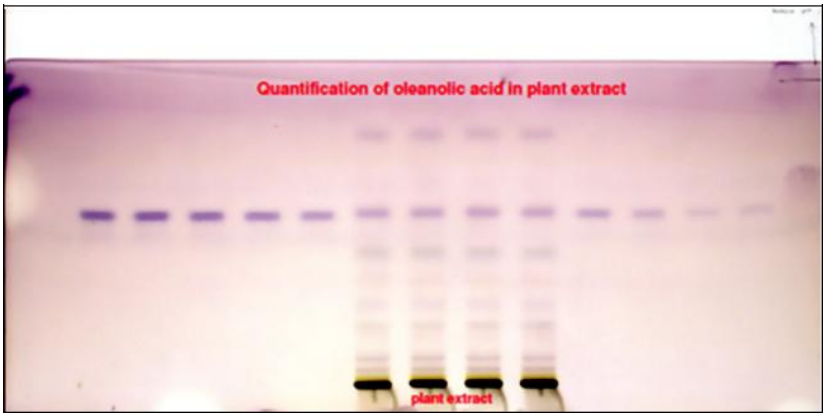


Plate 1. HPTLC profile of Quantification of Oleanolic Acid in Root extract of *Achyranthes aspera* Linn

4. CONCLUSION

The quantification of oleanolic acid from the methanolic extract of roots of *A. aspera* Linn. was done using a new HPTLC method. The developed HPTLC technique can be used for the routine quality control analysis and quantitative determination of oleanolic acid from *A. aspera* Linn. The oleanolic acid was found to be linear in the range of 0.20-0.60 µg/µl. Considering the wide therapeutic applications of oleanolic acid an alternative quantification technique of this marker constituent was generated to ensure identity and quality of the selected plant. This is a sensitive, specific and reproducible HPTLC method for the quantification of oleanolic acid from roots of *A. aspera* Linn.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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