DEVELOPMENT, EVALUATION AND CHARACTERIZATION OF NOVEL ANTI-VITILIGO OINTMENT FROM HIMALAYAN *Heracleum candicans* Wall. ex DC.

Thesis Submitted for the Award of the Degree of

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in

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DECLARATION

I, hereby declared that the presented work in the thesis entitled **"DEVELOPMENT, EVALUATION AND CHARACTERIZATION OF NOVEL ANTI-VITILIGO OINTMENT FROM HIMALAYAN *Heracleum candicans* Wall. ex DC."** in fulfillment of degree of **Doctor of Philosophy (Ph.D.)** is outcome of research work carried out by me under the supervision of **Dr. Iqubal Singh,** working as **Assistant Professor** in the **Department of Pharmaceutical Sciences** of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled "**DEVELOPMENT, EVALUATION AND CHARACTERIZATION OF NOVEL ANTI-VITILIGO OINTMENT FROM HIMALAYAN *Heracleum candicans* Wall. ex DC."** submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the **Pharmaceutical Sciences** is a research work carried out by **Nissar Ahmad Dar**, **41800248** is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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Nissar Ahmad Dar

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List of Abbreviation

AQbD	Analytical Quality by Design
BBD	Box-Behnken Design
BCIL	Biotechnology Consortium India Limited
CAAs	Critical Analytical Attributes
cfu/gm.	Colony Forming Units per Gram
CMPs	Critical Method Parameters
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
DPPH	1,1, Diphenylpicrylhydrazyl
FCCD	Fractional Factorial Central Composite Design
FRAP	Ferric Ion Reducing Anti-oxidant Parameter
HETP	Height Equivalent to a Theoretical Plate
HPLC	High-Performance Liquid Chromatography
ICH	International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
IAEC	Institutional Animal Ethics Committee
IUCN	International Union for Conservation of Nature
LOD	Limit of Detection
LOQ	Limit of Quantification
LQC	Lower Quality Control
MLRA	Multiple Linear Regression Analysis
MODR	Method Operable Design Region
MQC	Intermediate Quality Control
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OECD	Organization for Economic Cooperation and Development
ROS	Reactive Oxygen Species

ABSTRACT

Vitiligo is a skin disease affecting 1–2% of the global population, primarily due to the depletion of dermal pigment-producing cells. Melanocytes in vitiligo patients exhibit a higher vulnerability to external oxidative stress, leading to increased stress and reactive oxygen species quantities. Treatment options include Ayurveda, surgery, homeopathy, and conventional medicine. Anti-oxidants stabilize or neutralize free radicals, protecting cells and organs from oxidative stress-induced damage. The herbal medications having high levels of furanocoumarins and polyphenols, etc. have been utilized traditionally for their immunological, anti-inflammatory, and anti-oxidant effects.

This research aims to address the challenges related to vitiligo treatment by incorporating the herbal extract from *H. candicans*, a less explored Himalayan medicinal plant known for its traditional use against leucoderma from the Uttarakhand Himalayan region. Scientific research has found a number of chemicals in *H. candicans* that are useful for medicine.

The exploratory phytochemical assessment of different *H. candicans* extracts indicated a diversity of carbohydrates, flavonoids, phytosterols, coumarins, protein molecules, saponins, diterpenes, cardiac glycosides, lipids, and oils. The pharmacognostic evaluation of *H. candicans* established the existence of prismatic calcium oxalate crystals, anomocytic stomata, and simple starch grains, as well as two sorts of covering trichomes. The analysis indicated the existence of spiral and reticulated xylem vessels (lignified) as well as lignified fibers, which could be helpful in a precise recognition of the relatively unexplored crude drugs of plants.

The *in vitro* anti-oxidant potential of various *H. candicans* extracts of aerial portion was examined. The total phenolic content in different plant extracts was found highest in the ethyl acetate extract (68.17 mg/g) followed by the chloroform (53.91 mg/g) while as least content was seen in hydroalcoholic (34.5 mg/g). The DPPH radical-scavenging activity of the *H. candicans* aerial part of the plant showed the potent radical-scavenging properties, while the extract from hydroalcoholic showed the least. A positive correlation was found between the percentage of DPPH radicals scavenged and the

potency of anti-oxidant potential. The maximum overall level of flavonoid was identified in the chloroform extract (69.50 µg/ml) of the plant, followed by the petroleum ether extract of the plant, while the least flavonoid content was shown by the ethyl acetate extract (18.37 µg/ml) of the aerial part of the plant. The reducing potency of different extracts from the aerial parts of *H. candicans* was found highest in the methanolic, chloroform extracts and least potency was noted in the petroleum ether extract. The lipid peroxidation inhibition of different extracts of the aerial part of the plant inferred that the ethyl acetate and chloroform extracts have maximum inhibition of the lipid peroxidase while as hydroalcoholic extract showed the minimum ILP %.

MTT cytotoxicity studies lead us to infer that against B16F10 cell lines, samples, namely psoralen, xanthotoxin, chloroform extract, ethyl acetate extract, methanolic extract, and hydroalcoholic extract, show moderate cytotoxic potential properties with more than 75% cell viability at 50 µg/ml, respectively. Petroleum ether extract showed effective cytotoxicity on B16F10 cells, with an IC_{50} value of 34.49 µg/ml. Findings from the tyrosinase enzyme study's statistical assessment indicate a dose-dependent effect on tyrosinase activity for both psoralen and xanthotoxin and some plant extracts. Psoralen exhibits a steady increase in tyrosinase activity with increasing concentrations, reaching a maximum of 156.27% at 25 µg/ml. Similarly, xanthotoxin shows concentration-dependent increase, with a maximum activity of 147.18% at 25 µg/ml. Among the plant extracts, the chloroform extract demonstrates maximum activity of 138.74% at 25 µg/ml. In contrast, the hydroalcoholic extract shows a less pronounced effect. Ethyl acetate and chloroform extracts from the aerial parts of *H. candicans* have thus shown good anti-vitiligo properties. The data reveals concentration-dependent effects on melanin secretion by B16F10 cells in response to psoralen, xanthotoxin, and various plant extracts. Psoralen exhibits a dose-dependent increase in melanin content, reaching 184.90% at 25 µg/ml thus, indicating a potential stimulatory effect on melanogenesis. Xanthotoxin demonstrates a similar concentration-dependent pattern, with a maximum melanin content of 156.60% at 25 µg/ml. Also notably, xanthotoxin appears to induce melanin synthesis not quite as much as psoralen. Among the plant extracts, the chloroform extract

induces a significant increase in melanin content, reaching 155.03% at 25 μg/ml. This study confirms the traditional claim that the *H. candicans* plant is being used for anti-vitiligo, highlighting its potential in treating this disease.

The study also aimed to validate the *in vivo* anti-vitiligo capabilities of a chloroform extract and ethyl acetate extract derived from the plant *H. candicans*, which is known to contain furanocoumarins such as psoralens and xanthotoxin. The results showed that the chloroform extract, when locally applied in an ointment form at a 10% concentration for 50 days, led to a gradual disappearance of vitiligo on the skin of C57 mice. Estimation of N-acetyl-d-glucosamine levels data indicate that both chloroform and ethyl acetate extracts, when formulated as 10% ointments, showed potential anti-vitiligo effects, as evidenced by the reduced NAG concentrations compared to the monobenzone-treated group. According to the research, coumarin-containing plants and their derivatives have been used for a long time to restore skin colour. Herbal mixtures with chloroform extract that were meant to be applied to the skin were tested for colour, smell, loss on drying, irritation-freeness, pH, diffusion study, spreadability, extrudability, solubility, consistency, washability, and viscosity. The F2 formulation of the chloroform extract showed promising attributes for further development and consideration in topical applications. Further stability studies to assess the stability of herbal ointment combinations using various physico-chemical metrics were also conducted for the developed formulation. The outcome of the stability research implied that the F2 formulation of the chloroform extract was stable under the given set of temperature conditions.

CHAPTER 1 INTRODUCTION

Human beings have been extensively dependent on plant resources not only for sustenance, attire, and housing but also for a wide range of medications to alleviate a variety of health conditions since ancient times. Approximately 80% of the global population in developing nations is reported to incorporate herbal remedies into their healthcare practices (Djordjevic, 2017; Olsen, 1998). This extraordinary proportion is ascribed to quite a few aspects, including the inadequate availability, accessibility, and affordability of contemporary medications (Shewamene *et al*., 2017). The primary healthcare needs of the underprivileged in developing nations as well as those in nations where conventional medicine denotes backbone in the national healthcare institutions are met by the use of traditional medicines (Lanfranco, 1999). So far, almost very little has been done for the cultivation of innumerable herbs of medicinal importance. Without implementing a precise strategy for commercial-scale cultivation, valuable medicinal species may approach extinction (Pandey & Savita, 2017). Increasing demand from the pharmaceutical industry has made medicinal plants crucial for generating income through exports, emphasizing their industrial importance (Ganesan, 2008).

1.1 Vitiligo

Vitiligo a repugnant, terrible disease of the time with a debilitating condition that can cause individuals to undergo stigmatized and devalued due to its disfiguring nature. It detrimentally affects the alteration of human skin pigmentation arises from the depletion of dermal pigment-producing cells, impacting approximately 1-2% of global populace (Khandalavala & Nirmalraj, 2014). This condition known is a skin illness that is hereditary and has both medical and sociological ramifications. It is distinguished by the prevalence of depigmented macules on the skin, triggered by the impairment of pigment producing cells and the consequent suppression of melanin biosynthesis (Ezzedine *et al*., 2015; Rodrigues *et al*., 2017). Vitiligo manifests as an autoimmune dermal disorder affecting pigmentation, marked by the emergence of distinct depigmented patches on

both the skin and mucous membranes (Rodrigues *et al*., 2017). Vitiligo constitutes a persistent autoimmune onslaught on melanocytes, resulting in the depletion of pigmentation across the skin and mucosal surfaces, culminating in the progressive enlargement of depigmented skin plaques (Seneschal *et al*., 2021). Vitiligo represents an acquired medical and social pigmentary anomaly of the skin, distinguished by well-defined white patches or macules. These white patches are generated by the apoptosis of pigment cells and consequent suppression of melanin development (Ezzedine *et al*., 2015; Rodrigues *et al*., 2017).

1.2 Prevalence and social impact

The occurrence of vitiligo is commonly cited within the range of 0.5–2% across the global populace (Ezzedine *et al*., 2012). The etiology of vitiligo is posited as a multifaceted interplay involving genetics, environmental factors, oxidative stress, and autoimmune mechanisms (Laddha *et al*., 2013; Wang *et al*., 2016). Vitiligo typically starts in adolescence or adolescence deprived of ethnic improvement or sex (Halder & Chappell, 2009). While primarily devoid of physical symptoms, the socioeconomic repercussions of vitiligo potentially have substantial and far-reaching consequences and significantly affect individuals who are regularly in search for a powerful treatment. Its prevalence is varying from 0.46-8.8% in India. It is reported that as many as 65 million people suffer from vitiligo worldwide. Shockingly, India hosts 0.46-8.8% vitiligo patients against 0.5-1% of the world vitiligo population. Among the various states of country, Gujarat and Rajasthan scores the highest (Handa & Kaur, 1999; Valia & Dutta, 1996). In India, young ladies are progressively inclined to vitiligo when compared with that of young men with a predominance rate of 0.48% (Das *et al*., 1985; Handa & Dogra, 2003).

1.3 Pathogenesis of Vitiligo

Vitiligo's onset is believed to result from an intricate interplay involving oxidative stress, genetics, autoimmunity, and environment considerations (Laddha *et al*., 2013; Wang *et al*., 2016). The predominant conjecture, substantiated by robust evidence, postulates the autoimmune theory as the forefront explanation for the development of vitiligo

(Richmond *et al*., 2013). The prevailing hypothesis suggests that the malady principally stems from the annihilation of melanocytes and impediment of melanin creation pathway (Boniface *et al*., 2018; Ralf Paus *et al*., 2008). Melanin is integral to the skin's pigment system, playing a vital role in shielding against harm from ultraviolet light (Gupta *et al*., 2006). Melanocytes produce melanin through a process known as melanogenesis within the melanosome (Yamaguchi & Hearing, 2014). Melanogenesis, a vital physiological route, is responsible for generating the melanin pigment—a crucial step in regulating melanocyte functions, notably for photo protection (Pillaiyar, Manickam, & Jung, 2017). Melanogenesis, the process of melanin production, is governed by particularly significant enzymes, chiefly tyrosinase linked protein 1 (TRP 1), tyrosinase (TYR), and tyrosinase associated protein-2, possess a critically important role (Niu & Aisa, 2017a). Tyrosinase directly controls melanin production by oxidizing melanogenesis associated substrates like tyrosine (Kim & Uyama, 2005). The Microphthalmia associated transcriptional variable, functions primarily as a key regulator, synchronizing the expression of genes critical for melanin synthesis (Levy *et al*., 2006).

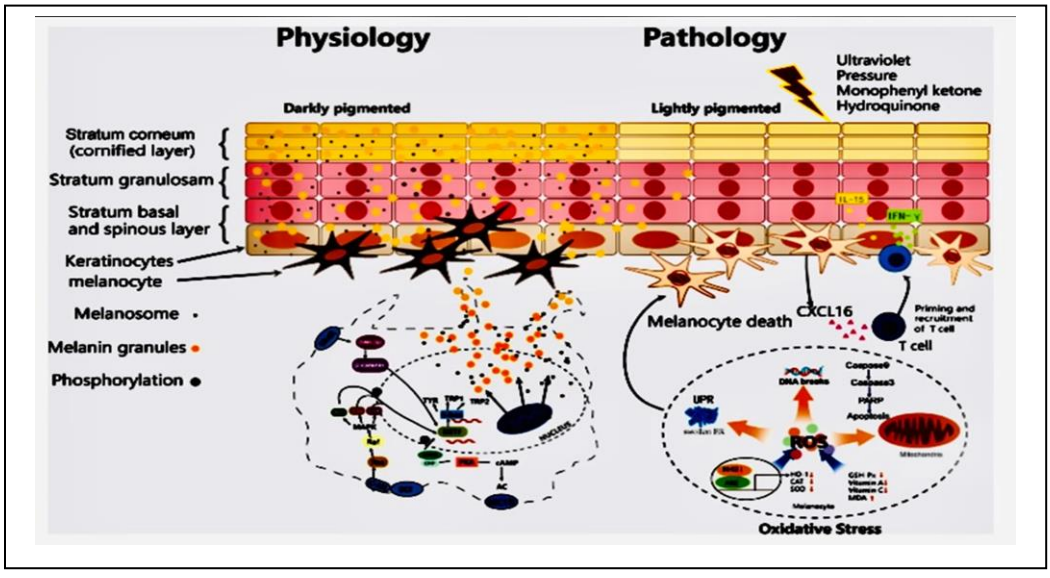


Figure 1: Pathophysiology of melanocytes in vitiligo (Pang *et al*., 2021).

The etiology of the disease is intricate and necessitates the concurrent involvement of melanin-producing cells, naïve lymphatic cells, fibroblasts, keratinocytes, and natural

killer lymphocytes (Seneschal *et al*., 2021). Therapeutically, it is identified into three distinct sections: segmental, non-segmental, and hybrid pigmentation disorder (Ezzedine *et al*., 2015). Vitiligo of non-segmental type is the primarily prevalent in nature, characterized by distinct boundaries, reticular patterns, varied sizes, distributions, depigmentation, and a milky-white appearance. Segmented vitiligo, which accounts for 5-16% of incidents, appears as areas on one side of the body, whereas non-segmental vitiligo contains both regions across one side and spots on both sides of the body (Ezzedine *et al*., 2015; Speeckaert *et al*., 2020). Several internal mechanisms, are involved including cell growth, production of melanin, immune system activity, cellular energy processes, cell specialization, and programmed cell death (Al-Shobaili & Rasheed, 2015). Denat *et al*. (2014) provide well-built argument that oxidant stress plays a crucial role during the development and advancement of illnesses (Denat *et al*., 2014). Cellular oxidation stress, derived from combined intrinsic and exogenous factors, prompts immunological processes in melanin cells which are correlated to the condition (Abdel-Malek *et al*., 2020; Chen *et al*., 2019; Xie *et al*., 2016). Increased upsurge in radical oxide molecules is an outcome of reduced scavenger activities, primarily in catalase and glutathione peroxidase. The dearth of anti-oxidants in melanin cells makes them susceptible to increased deposition of radical oxygen ions, resulting in compromised mitochondrial functioning, DNA destruction, lipid oxidation, and protein degradation (Bickers & Athar, 2006; Chen *et al*., 2021).

1.4 Treatment Options and Future Prospects

The primary aim of addressing vitiligo is to manage its progression and attain re-pigmentation. Typically, controlling the spread of vitiligo requires a prolonged time frame. Given the lack of clear understanding regarding the precise cause in each case, achieving desired success in treatment can be challenging. Current treatment choices encompass Ayurveda, surgery, homeopathy, additional therapies and conventional medicine. There exists significant potential for the development of innovative and more effective treatment approaches for vitiligo. Current medications for vitiligo treatment,

dermal autoimmune suppressors, radiation therapy, as well as prudent approaches somewhat meet the aforementioned goals in a general but non-specific manner. These options can be categorized into groups such as immunomodulators; dermal steroid medications, calcipotriol, pseudo catalase, oral medicines, synthetic vitamin D analogs, light therapies, surgical approaches, and discoloration are treatment options available for this condition. Despite the array of treatment approaches, it is often problematic and disappointing both for the patients as well as the specialists (Soni *et al*., 2010). No FDA authorized interventions from medicine for vitiligo is available so far (Rashighi *et al*., 2014). Regrettably, the current medical treatments for this condition do not yield sound therapeutic responses, leading to an increasing inclination towards complementary medicine among vitiligo patients (Yoon *et al*., 2011). A successful therapy for vitiligo hinges on either boosting the appearance of typical melanin cells in vitiliginous spots or the spread and propagation of pigment-producing cells in the adjacent tissues. Furthermore, the acquisition of melanin by epithelial tissues relies on the capability of active melanin-producing cells (Spritz, 2011).

A satisfactory cure for vitiligo is currently elusive. Melanocytes in the skin of vitiligo patients, in contrast to routine melanin cells, exhibit higher vulnerability to exogenous stress from oxidation (Jimbow *et al*., 2001; Maresca *et al*., 1997). This heightened susceptibility leads to heightened concentrations of reactivity in oxygen moieties and elevated stress in endoplasmic reticulum (Karin U Schallreuter *et al*., 1999b; Shalbaf *et al*., 2008). These consequences can be mimicked by exposure of phenolic compounds to the skin which include hydroquinone monobenzyl ether (i.e. monobenzone) and 4-tert-butylphenol, exacerbating the clinical depigmentation observed in vitiligo patients (Rattan *et al*., 2011).

Currently, various pharmaceuticals are employed during the management of pigmentation disorder, encompassing photo sensitizers; khellin and 8-MOP, calcineurin inhibitors like tacrolimus, vitamin D_3 analogs exemplified by tacalcitol, and topical corticosteroids such as dexamethasone (Felsten *et al*., 2011), and JAK inhibitors (baricitinib, tofacitinib, ruxolitinib), are the agents commonly prescribed for myelo-

dysplastic disorders have recently demonstrated efficacy in treating vitiligo, as evidenced by a substantial number of clinical cases (Damsky & King, 2017; Harris *et al*., 2016). Plant extracts derived from *Psoralea corylifolia* has historically been employed for repigmentation in vitiligo, coupled with exposure to natural sunlight. The active constituents, furocoumarins, specifically 5-methoxypsoralen, 8-methoxypsoralen, and 4, 5, 8-trimethylpsoralen, have been clinically utilized. Subsequent investigations have substantiated their robust photosensitivity properties (Fowlks *et al*., 1958), this could potentially confer advantages for skin repigmentation upon subsequent exposure to long-wavelength ultraviolet radiation (Parrish *et al*., 1974). Despite encountering certain undesirable side effects (Felsten *et al*., 2011; Tippisetty *et al*., 2013), nevertheless, the therapy remains the most efficacious approach for treating the disease at present. Potential targets of furocoumarin in these types of therapeutics remain ambiguous owing to the complexities of the disease's progression, and only a limited number of derivatives with anti-vitiligo activity have been documented (Guiotto *et al*., 1984; Matsuda *et al*., 2005a).

1.5 Herbal Medicine and Vitiligo

Significance of plant-derived medicines is genuinely underestimated in modern medicine. A growing majority of botanicals are acknowledged as a large treasure of natural phytochemicals which possess substantial biological activities. A variety of medicinal plant components are employed to extract raw drugs, each harboring distinct curative properties (Egwaikhide & Gimba, 2007; Gisesa, 2004). India holds a wealth of meticulously documented and traditionally accomplished data in herbal medicine. It stands as one of the foremost producers of medicinal herbs and is aptly regarded as the "*Botanical Garden*" of the world. Based on local flora, culture, and religion, traditional medicine has advanced over the centuries (Bandaranayake, 2006; Cassileth, 1998; Cragg & Newman, 2001; Lans *et al*., 2001). The exploration of active compounds within plants has unveiled new medicinal drugs, demonstrating both proficient protective and treatment properties against diverse diseases (Roy *et al*., 2010). Only approximately 15% of the Angiosperms (flowering plants) have been chemically investigated for their therapeutic

potential (Farnsworth, 1966; Farnsworth & Soejarto, 1991). Diverse components of medicinal plants are employed for extracting raw drugs, each harboring a variety of therapeutic properties (Egwaikhide & Gimba, 2007; Gisesa, 2004). Several studies have demonstrated that plant phytochemicals could be employed as therapeutical benefit for management of diseases (Soylu *et al*., 2010). Multiple variables influence to this expansion, comprising consumer preference for natural remedies, apprehensions about adverse effects associated with modern medications, substantiated evidence of efficacy, heightened interest in alternative medicines, the perception that natural medicines are free of adverse outcomes, a population inclination toward preventive healthcare due to aging demographics, a proclivity for self-medication, advancements in the quality and safety of herbal medicines, and the elevated cost of synthetic pharmaceuticals (Ceylan & Fung, 2004; Rattanachaikunsopon & Phumkhachorn, 2010).

Multiple researchers have examined the utilization of therapeutic herbs in vitiligo management, the available evidence supporting their effectiveness in this regard remains limited (Fisk *et al*., 2014; Szczurko & Boon, 2008). In the management of vitiligo, herbal medicine, alongside alternative treatment modalities, has been employed. Specifically, herbs rich in furanocoumarins, including Khellin, naphthodianthrones, Polyphenols, *Hypericum perforatum*, and other major coumarin or furanocoumarins rich botanicals have been utilized (Amro *et al*., 2021; Shakhbazova *et al*., 2021). These therapeutic interventions appear to be associated their natural photosensitizing capabilities along with their immunological, anti-inflammatory, and anti-oxidant characteristics (Gianfaldoni *et al*., 2018). In addition, certain compounds have been discovered to function as inducers of tyrosinase, contributing to enhanced pigmentation. Notably, extracts from *Daphne gnidium*, encompassing essential oils with fatty acids, terpenoids, flavonoids, coumarins, and alkanes, exhibit this inducible quality. Similarly, *Moricandia arvensis* leaves, rich in tannins, saponins, cardenolides, alkaloids, and flavonoids have also been identified as agents that stimulate tyrosinase activity to promote pigmentation (Berreghioua & Cheriti, 2018). *Vernonia antheimintica*, according to findings encompasses a combination of steroids, fatty acids, flavonoids, carbohydrates, terpenes, and sesquiterpene lactones

(Dogra *et al*., 2020). Skin repigmentation has a long history in Egypt and India, namely with plants and their derivatives that possess coumarins, such as *Ammi majus*, *Psoralea corylifolia*, *Ficus carica* (Niu & Aisa, 2017b). Moreover, scientific literature suggests that psoralens or linear furanocoumarins serve as photosensitizing agents, heightening skin sensitivity and facilitating increased melanin production. When employed with the phototherapy, these compounds have been documented as therapeutically effective in addressing vitiligo, showcasing their potential in augmenting the skin's responsiveness to treatment and promoting pigmentation (da Silva *et al*., 2009). Moreover, numerous studies confirm the significant photosensitivity of coumarins. This attribute holds promise for enhancing vitiligo treatment when coupled with ultraviolet radiation exposure, indicating a potential synergistic effect that merits further exploration in optimizing the therapeutic outcomes (Niu & Aisa, 2017a).

Research undertaken both *in vivo* and *in vitro* have found that natural substances stimulate the production of melanin and shield it from degradation through various mechanisms. Earlier research has consolidated three primary pathways in melanin biosynthesis regulated by tyrosinase: the cyclic AMP/PKA pathway, the mitogen-activated protein kinase, and the signaling Wnt/β-catenin pathway (Niu & Aisa, 2017a; Pillaiyar, Manickam, & Namasivayam, 2017). Within the screened compounds including, afzelin, quercetin, geniposide, puerarin, and others, were identified to promote melanocyte generation through modulation of the aforementioned pathways. In the light of the reported claim of anti-vitiligo potential of the *Heracleum candicans*, this research is intended to instill a ray of optimism in those feeling disheartened, through the utilization of a dosage form infused with herbal extract from *Heracleum candicans* in a suitable dosage form i.e., ointments. *Heracleum candicans*, a less explored Himalayan plant is claimed to possess anti-vitiligo properties and the plants has been found to contain coumarins. Coumarins, naturally derived compounds from plants, exhibit cytotoxic, immunomodulatory, and anti-oxidant properties. Numerous studies have documented the induction of repigmentation through the topical application of

coumarins. This effect is believed to influence immune system modulation when combined with trimethylpsoralens, (Conforti *et al*., 2009).

1.6 Vitiligo and oxidative stress- a correlation

The reactive oxygen species model posits that overabundances of reactive molecules are generated due to a malfunction in oxygen metabolism, leading to the destruction of functional melanocytes. Various neurogenic factors act through cytotoxic and immunological systems, causing melanocyte destruction and subsequent skin depigmentation. The onset of vitiligo can be triggered by various factors, such as sunburn and exposure to phenolic synthetic compounds, although the trigger remains unknown in many cases. Overall, these triggers are thought to encourage oxidative stress in melanocytes (Picardo & Bastonini, 2015). Individuals diagnosed with vitiligo have been observed to display impaired anti-oxidant responses. This suggests a potential deficiency in the body's ability to counteract oxidative stress, that may promote the advancement or emergence of the disorder (Yildirim *et al*., 2004). Oxidation distress promotes the aggregation of misfolded peptides throughout the endoplasmic membrane lumen, which leads to the triggering of the unfolded polypeptide reaction that reinstates physiological equilibrium and supports the survivability of the cell. Cellular oxidative damage is able to promote a perturbation in subsequent proportions of calcium ions inside the endoplasmic reticulum, therefore triggering the damaged polypeptide responses and the induction of cellular death (Carreras-Sureda *et al*., 2018). Reactive stress triggers the unfolded protein response, which induces immunological responses and boosts CD8+ T cells. The activity leads to the removal of epidermal melanin cells as a byproduct of a strong feedback cycle comprising pro-inflammatory mediators (Bergqvist & Ezzedine, 2021).

Moreover, there is often a presence of comprehensive oxidative stress reported in vitiligo (Guerra *et al*., 2010; Laddha *et al*., 2013; Shah & Sinha, 2013). Likewise, an earlier study also proposed oxidative stress as the primary trigger for initiating vitiligo (Laddha *et al*., 2013). Therefore, stress due to oxidants has been established as the principal triggering cause in vitiligo (Glassman, 2014; Laddha *et al*., 2013). Anti-oxidant intervention is widely seen as a potential means to mitigate oxidative stress, playing a

pivotal role in preventing and treating vitiligo (Akoglu *et al*., 2013). In vitiligo, there are heightened concentrations of oxidatives like superoxide dismutase observed in both perilesional tissue and sera, indicating an intricate relationship between oxidative damage and the corresponding anti radical scavenging actions. Further exploration is needed to unravel the mechanisms and implications of these elevated enzyme levels (Dammak *et al*., 2009). A candidate gene association study in the Chinese populace has uncovered a substantial correlation between a single nucleotide polymorphism within NRF2 and vitiligo, suggesting the pivotal role of anti-oxidants in the condition (Song *et al*., 2016). Melanocytes are more susceptible to oxidative stress damage than keratinocytes and fibroblasts (Hoogduijn *et al*., 2004; Liu *et al*., 2009; Valverde *et al*., 1996). Prior research suggests that oxidative stress is a significant factor in melanocyte loss (Maresca *et al*., 1997; Schallreuter *et al*., 1999b).

In light of the above-mentioned problems with respect to vitiligo treatment, this research is intended to bring a ray of expectation to the "down casted" by a dosage form incorporated with the herbal extract from *Heracleum candicans* in a suitable dosage form i.e., ointments. The plant *H. candicans* is a less explored Himalayan medicinal plant which is reported for its traditional use against leukoderma from Uttarakhand Himalayan region and found to contain coumarins. Coumarins, natural compounds with cytotoxic, immunomodulatory, and anti-oxidant properties, have been reported in numerous studies to induce repigmentation upon topical application. This is believed to influence immune system modulation when combined with trimethylpsoralens (Conforti *et al*., 2009).

Hence, the hypothesis was made that the *H. candicans* could possess significant anti-vitiligo properties and a suitable formulation could lead to the societal benefit at large. Therefore, the objective of the current research is to explore the anti-vitiligo and anti-oxidant capabilities of *H. candicans* extract in a suitable dosage form.

CHAPTER 2 REVIEW OF LITERATURE

Review of literature will consist of the following points.

I. Preliminary details of herbs viz., taxonomical data, vernacular names, geographical distribution, and parts used chemical nature and constituents, traditional uses and
II. Reported pharmacological activities of *Heracleum candicans*

2.1 Heracleum genus

Naturally derived pharmaceuticals have stirred up substantial interest as a result of their extensive spectrum of possibilities for therapeutics. Medicinal plants represent a prolific source of compounds utilized in traditional medicine, contemporary pharmaceuticals, dietary supplements, nutraceuticals, pharmaceutical intermediates, folk remedies, and synthetic drug components (Ncube *et al*., 2008). The Apiaceae family, previously recognized as Umbelliferae, stands among the spectrum of Angiosperms, renowned for its multitude array of medicinally valuable species (Rawat, 1988). With approximately 450 genera and 3700 species globally, it is a significant and varied plant family (Pimenov, M & Leonov, 1993). Among the 186 species in this family, 80.60% of species are of Himalayan origin (Prasanta Kumar Mukherjee, 1982). As per Samant *et al*. (1998), more than a quarter of the total representatives from the Apiaceae family in the Indian Himalayas hold curative significance (Samant *et al*., 1998). The majority of plants in the Apiaceae family are aromatic, characterized by hollow stems, commonly referred to as umbellifers. They are utilized as traditional ethno medical remedies, and there are records of over 100 cultivated species with various applications (Yoganarasimhan & Chelladurai, 1996). This family is typically identified by alternative leaves that have base-widen, the stem includes a furrowed coating. Flowers tend to be multipart and clustered under umbrellas having five asymmetric petals and five stamens, usually typical. The fruit has

bivalent, monospermous sections that split when mature. Certain plant components typically emit a potent fragrance, mainly attributed to diverse oil-producing glands.

2.1.1 Diversity and distribution of Heracleum genus

The wide-ranging distribution and taxonomic intricacy of Heracleum species characterize this genus, belonging to the Apiaceae family's subfamily Apioideae. The Latin suffix "Heracleum" originated out of the Greek "Herâclêus," denoting the "glory of Hera" and represents an aspect of the Herculean family. There are almost 125 Heracleum species in the world (Pimenov & Leonov, 2004) and its primary centers of diversity Caspian and Sino-Himalayans Mountains (Logacheva *et al*., 2008; Ma *et al*., 2005; Pimenov *et al*., 1993; Pu, Fading; Watson, 2005). Genus Heracleum is a perennial, distributed in Africa, Europe, Asia, and North America (Aswal & Mehrotra, 1994). This genus is widely distributed in Asia (Pimenov & Leonov, 2004). India is habitat to above 15 different species, five are exclusive to the Coromandel constituency (Kaul, 1989; Nayar, 1996). In the late 19th century, Drude (1897–1898) categorized the Heracleum genus within the Apiaceae tribe Peucedaneae, sub tribe Tordyliinae. Later, Pimenov and Leonov (1993) acknowledged Heracleum in the Tordylieae tribe, and subsequent phylogenetic analyses of molecular data have consistently supported its placement within this tribe (Ajani *et al*., 2008; Downie *et al*., 2001). At first, the bunch was deemed as the *''Heracleum clade''* (Downie *et al*., 2000; Downie *et al*., 2001), it has also been regarded as the monophyletic genus while the Tordylieae sub-tribe is recognized by Tordyliinae (Downie *et al*., 2010). The Heracleum genus, within the broader family, is globally renowned for its widespread traditional and contemporary applications. Although Heracleum has historically been acknowledged as innate group and the molecular phylogeny investigations revealed its non-monophyletic nature (Ajani *et al*., 2008; Downie *et al*., 1998, 2000, 2010; Katz-Downie *et al*., 1999; Logacheva *et al*., 2008; Zhou *et al*., 2008, 2009). In traditional medicinal practices, certain Heracleum species are employed for their analgesic, antipyretic, and diaphoretic properties (Taniguchi *et al*., 2005), anti-septic, digestive, carminative, and food condiment (Sonboli *et al*., 2007; Souri *et al*., 2004), for gastralgia, lumber pain, rheumatic disease, fracturing as well as injuries associated with falls,

bruise/trauma and tendonitis (Niu *et al*., 2004) and in the management of elevated blood pressure (Eddouks *et al*., 2002), epileptic seizures (Eadie, 2004; Sayyah *et al*., 2005) and for crippling weakness, therapy for arthritis (Chacko *et al*., 2000), gastritis, diarrhea (Baytop, 1999). The aromatic oils derived from Heracleum genus have undergone comprehensive exploration for their anti-fungal, anti-dermatophytic, anti-bacterial, and insecticidal properties (Cieśla *et al*., 2008; Jagannath *et al*., 2012; Özçakmak, 2012; Özkırım *et al*., 2012). The entire genus is abundant in furanocoumarins, undergoing a chemical transformation into xanthotoxin (Handa, 1970). Also, certain species are utilized in various industries like as nourishment, spices, livestock feed, sauces, and perfumes (Kaul, 1989). Heracleum species has been explored for aromatic oils of their functional parts (Borg-Karlson *et al*., 1993). Different Heracleum species yield a diverse array of derived metabolites, like furanocoumarins, furanocoumarin dimers, hydrocarbons, flavonoids, anthraquinones, stilbenes, oxygenated monoterpenes, and sesquiterpenes (Kuljanabhagavad *et al*., 2010).

Table 1: Heracleum diverse species throughout India (Hooker, 1879).

India's north and north-eastern areas	South India and Ceylon areas
Heracleum canascens	*Heracleum aquilegifolium*
Heracleum barmanicum	*Heracleum ceylanicum.*
Heracleum brunonis.	*Heracleum concanense*
Heracleum cachemiricum	*Heracleum hookerianum*
Heracleum candicans	*Heracleum panda*
Heracleum jacquemontii	*Heracleum pedatum.*
Heracleum nepalense	*Heracleum rigens*
Heracleum nubigenum	*Heracleum sprengelianum*
Heracleum obtusifolium	
Heracleum pinnatum	
Heracleum sublineare	
Heracleum thomsonii	
Heracleum wallichii	

2.1.2 Distribution, economic potential, and population status of Heracleum candicans

H. candicans, an indigenous species of the Himalayas, is wide spread in the mountainous and alpine areas of south western China, western Pakistan, Bhutan, Afghanistan, Nepal, and northern India. *H. candicans* Wall. ex DC., a member of the Heracleum genus that has been subjected to molecular scrutiny to date, exhibits genetic distinctiveness when compared to other congeners within its genus, including the prototype species *Heracleum sphondylium* (Ajani *et al*., 2008; Downie *et al*., 2000).

Kingdom	Plantae
Phylum	Tracheophyta
Subphylum	Magnoliopsida
Order	Apiales
Family	Apiaceae
Genus	Heracleum
Species	*Heracleum candicans*

H. candicans Wallich ex de Candolle holds substantial significance in economics as a foremost source of xanthotoxin, a compound crucial in the formulation of sun block creams endowed with anti-leucodermal properties. Xanthotoxin is broadly employed in the management of leucoderma and as a key component in sun-tanning lotions. Derived from diverse plant species within the genus Heracleum, this substance has been obtained not only from the Himalayan locale but also from various other regions. Among the Himalayan species, *Heracleum pinnatum* contains the lowest concentration of xanthotoxin at 0.005%, followed by *Heracleumc achemiricum* at 0.05%, and *Heracleum candicans* at 1.5% has the highest content (Banerjee *et al*., 1979; Kaul, 1989). Owing to its substantial xanthotoxin composition, there is a pronounced inclination for this botanical specimen within the pharmaceutical sector, establishing it as a pivotal component among India's exported medicinal plants (BCIL, 1996). As per Kaul's (1989) study, an annual harvest of more than 150 metric tons of recently cultivated plant roots

was documented from untamed resources in the Himalayan expanse of Kashmir during the period spanning from 1980 to 1985.

Table 2: Geographical dispersion of *Heracleum candicans* (Butola *et al*., 2010)

World	Bordering elevations of 1800-4500 m asl in western Pakistan, Bhutan, Nepal, Afghanistan, and southwestern China
India	North Indian states of Jammu & Kashmir, Uttarakhand, Sikkim, and the Himachal Pradesh region.

Table 3: Geographical dispersion of *Heracleum candicans* over many states in India (Butola *et al*., 2010)

Region	Distribution Range (m)	Average Density (ind./m^2)	Districts (Locality)
Himachal Pradesh	1800-5000	1-1.65	**Kinnaur:** Rispa, Charming Sarahan-Chora, Sangla Valley **Kullu:** Banjar valley with Jalodi Pass, Kanawar Wildlife Sanctuary, Renowned Rohtang pass, Malana, and the distinguished Manali Wildlife Sanctuary, Hirb and Shojha catchments, Parvati Valley featuring, Acclaimed Great Himalayan National Park alongside Khokan Wildlife Sanctuary **Lahaul and Spiti:** Trilokinath, Khoksar, Gosal, Tindi, Mulling, Yangla, Hinsha, Bihadi, Nalda, Scenic Pin Valley **Mandi:** Kamrunag, Jaidevi Forest Division, Sanctuary of Nargu, Churag Valley, Jwalapur **Chamba:** Dharwas Valley, Pangi Valley, Bharmour **Kangra:** Titarcha and Treuend Hills **Shimla:** Mahaso, Rohru Forest Division
Uttarakhand	2000-4000	1-2	**Almora:** Doonagiri, Morunala Reserve Forest Near Bhimtal **Bageshwar:** Pindari Valley **Chamoli:** Bednibugyal, Kunwari Pass, Rudranath, Auli, Lata, Garpak, Bakkibugyal, Nanda Devi **Dehradun:** Mussoorie **Nainital:** Tipin Top, Mukteshwar **Pithoragarh:** Ghandhuru **Pouri:** Binser Forest (Chakisain), Bharsar

			Tehri: Panwali Kantha **Uttarkashi:** Harkidun
Jammu and Kashmir	1700-3100	2-3	**Bandipora:** Guraz Valley **Baramulla:** Tangmerg, Uri, Drung, Gulmerg **Gandbral:** Dachigam Sanctuary, Boniyar**,** Harwan**,** **Jammu:** Khan *et al*. (2009) found that the species is confined to 800–1500 m in the Sewa river watershed. This contrasts prior findings of 1700-5000 m distribution. **Kargil:** Kargil **Kupwara:** Sadhna valley, Lolab valley, Budinimal, Machile, Bungus, Rajwar, Keran **Leh:** Drass, Ladakh
Sikkim	2000-3500	1-2	Kangchenzonga Biosphere Reserve

The absence of standardized methods for propagating and cultivating *H. candicans* plants is a significant factor contributing to this problem. In the past, there were only a limited number of studies conducted on the phytochemistry, development, and agricultural technologies (Bhat & Kaul, 1979; Joshi & Dhar, 2003; Meena Joshi *et al*., 2004; Kaul, 1989).

Table 4: Overview of *Heracleum candicans* (Butola *et al*., 2010)

Aspect	Details
Taxonomy	Species with synonymous or taxonomically similar classifications include *H. lanatum, H. nepalense*, and the variety *H. obtusifolium*
Local Names	In Nepal: Tukar, Sukar, and Chhetaro In Pakistan: Folla In Andhra Pradesh and Tamil Nadu: Hakh Bul In Himachal Pradesh: Patishan, Patlain, Patrala, Rasal, Tukar, Gojihwa, Tunak, Padara, Radara In Uttarakhand: Arwa, Kakriya, Raswal In Kashmir: Ramthianthen, Patrali, Krandel, Gurkrandal, Hirwi, and Hirakali In English: Hogweed
Trading Name	Patrale, Patishan roots, Heracleum
IUCN Threat Status	Vulnerable in the Indian Himalayas range, notably in the northwest Himalaya, with a population decline of at least 50% in past decade
Cultivation Status	Undergoing investigational testing in a few national research institutions

Habitats	Inhabits exposed hillsides, grassy pastures, rugged recesses, streams, riverbanks, dense shrubberies, undulating swampy territories, and regions adjacent to farms. Altitude spans 1800 to 4300 m amsl.
Phenology	Blooms from June to July, yields fruit in August to September, and undergoes senescence from October to November.
Active Ingredients	Roots contain a high concentration (6-16%) of furanocoumarin, including 8-geranoxypsoralen, heraclenol, imperatorin, bergapten, and heraclenin. Other components include candicanin, sphondin, xanthotoxol and candicopimaric acid. Additionally, a chemical process converts a portion (1.5%) to xanthotoxin. The roots also yield approximately 0.1% essential oil.
Indigenous Uses	Traditional herbal cures for toothaches, arthritis, and liver issues. Young leaves and tender shoots are feed, while root paste treats dermatitis, skin problems, rashes, and joint pain. Fever, blood pressure, leprosy, earaches, stomach issues, infections, bleeding, and phlegm are treated with the herb. The fruits are used to spice and flavor food, and the seeds alleviate digestive and gas difficulties.
Pharmacological Uses	Fruits -pubescent, oval-shaped, and flattened are utilized as a nerve tonic and aphrodisiac. Root parts have antibacterial and anti-inflammatory effects. Xanthotoxin from roots used in vitiligo therapy and sun-tan
Toxicity/Taste/Potency/	Slightly toxic- Bitter and acrid taste - Neutral potency

2.1.3 Ethnobotany

Ethnobotany involves the examination of plants concerning the inhabitants of a specific region, delving into folk concepts of classification based on usage, habitat, habit, or other relevant parameters (Berlin *et al*., 1973). *Heracleum candicans* fruit powder is considered as aphrodisiac (Kapahi *et al*., 1993), is exploited in the ethnic system of medicine (Kritikar & Basu, 1993). Fruits are used as nerve tonic, and for intestinal parasites. The plant serves various purposes, including the treatment of earaches, expectorant and wind disorders, leprosy, blood loss, fever resulting from wounds, and blood pressure issues. Moreover, the leaves are employed as premium fodder, and the young aerial parts are palatable (Butola *et al*., 2010), Leucoderma, menstrual disorders (Joshi & Tyagi, 2011).

2.1.4 Phytochemical investigations

Wang *et al*., 2008 extracted three coumarins from *H. candicans*, namely heraclenin, 8-geranyloxypsoralen, and Imperatorin and were identified through bioassay-guided fractionations led by Wang and co-workers based on spectral data (Wang *et al*., 2008).

Nakamori *et al*., 2008 extracted novel compounds from *H. candicans* roots, including two coumarins candinol A and isophellodenol C, four spiro bifuranocoumarins (candibirins B-E), and two tri-furanocoumarins- canditririn-A and canditririn-B. Structural elucidation employed chemical and spectral techniques (Nakamori *et al*., 2008).

Inoue *et al*., 2010 obtained two novel ester coumarins, namely candinols B and C, and three coumarin dimers, identified as candibirins F-H from *H. candicans* roots. Structural determination involved chemical and spectral techniques (Inoue *et al*., 2010).

Taniguchi *et al*., 2011 isolated from *H. candicans* roots were three distinct spiro-trifuranocoumarins (canditririns C-E), spiro-tetrafuranocoumarin (canditetrarin A), and a novel tetra-furanocoumarin (canditetrarin B). Their structures were determined through spectral methods (Taniguchi *et al*., 2011).

Bogucka and Krzaczek, 2003 isolated from a crystalline furanocoumarin precipitate four compounds—bergapten, isopimpinellin, pimpinellin, and sphondinin. These compounds were identified through H1NMR analysis (Bogucka-Kocka & Krzaczek, 2003).

Kavli *et al*., 1984 isolated furocoumarins (psoralens) including bergapten, isobergapten, sphondin, isopimpinellin, and pimpinellin from the Umbelliferous plant Heracleum which were obtained through column chromatography. The structures and absorption spectra of these five furocoumarins were described (Kavli *et al*., 1984).

Doi *et al*., 2004 isolated a furanocoumarin dimer candibirin-A from *H. candicans* (Doi *et al*., 2004).

Inoue *et al*., 2010 during studies on roots of *H. candicans* yielded three novel coumarin dimers (candibirins F–H) and two coumarins (candinols B and C). Structural

determination employed a combination of spectroscopic and chemical methods (Inoue *et al*., 2010).

Taniguchi *et al*., 2011 during studies, discovered spirotri-furanocoumarin scanditririns C–E, spirotetra-furanocoumarin canditetrarin A, and tetra-furanocoumarin canditetrarin B from *H. candicans* roots, determined using spectroscopic techniques (Taniguchi *et al*., 2011).

Kunal *et al*., 1984 in assessment presented a comprehensive overview of the chemical constituents (coumarin, furanocoumarins, and other compounds) present in *H. candicans*. It showcased the abundance of fatty acids, coumarin, furanocoumarins, and their derivatives within this plant species.

Handong *et al*., while chromatographing the ether extract of dried roots of *H. candicans* over alumina revealed the presence of five known furanocoumarins (xanthotoxin, bergapten, heraclenin, beraclenol, isopimpinellin) and one known simple coumarin (suberosin). Additionally, the ^{13}C NMR spectral data for xanthotoxin, suberosin, and heraclenin were presented (Handong *et al*., 1984).

Inoue *et al*., 2011 found that the roots of *H. candicans* contains four new furanocoumarin glycosides, candinosides A, B, C, and D, were identified. Chemical and spectral methods were used to establish their respective structures (Inoue *et al*., 2011).

Wang *et al*., 2008 through fractionations of the *H. candicans* extract, three coumarins were isolated via bioassay-guided, identified as imperatorin, 8-geranyloxypsoralen, and heraclenin based on spectral data. All three compounds demonstrated nematicidal properties (Wang *et al*., 2008).

Bandopadhyay *et al*., 1971 obtained candicanin, a novel bi-coumarinyl derivative from the roots of *H. candicans* (Bandopadhyay, 1971).

Bandopadhyay, 1973 isolated from *H. candicans* roots: candicopimaric acid: a diterpene acid (Bandopadhyay, 1973).

Sharma *et al*., 1964 *H. candicans* was found to produce a novel furanocoumarin identified as heraclenin. The elucidation of its structure revealed it to be 8-(β,γ-oxido-isoamyloxy)-psoralen, marking a distinctive addition to the chemical profile of this plant species (Sharma *et al*., 1964).

2.1.5 Pharmacological investigations

2.1.5.1 Nematicidal Activity

Wang *et al*., 2008 found that *H. candicans* root extract exhibited antagonistic effects against *Panagrellus redivivus* and *Bursaphelenchus xylophilus* nematodes. The bioassay-guided fractionation isolated three coumarins-imperatorin, 8-geranyloxypsoralen, and heraclenin- each demonstrating nematicidal properties. This discovery marked the first report of nematicidal activity within the Umbelliferae family.

2.1.5.2 Antimicrobial Activity

Kaur *et al*., 2006, Kaur *et al*., 2010, Kaur *et al*., 2008, showed that the petroleum ether extracts from the roots of *H. candicans* demonstrated inhibitory effects specifically against *Pseudomonas* species and *Salmonella typhi*. In contrast, the chloroform and methanol extracts from both the root and shoot of the herb exhibited anti-bacterial potency against *Klebsiella, Escherichia coli*, and *Pseudomonas* species. Moreover, six distinct varieties of fungi exhibited sensitive to the anti-fungal properties of the chloroform extract of root and petroleum ether extract, while the petroleum ether shoot extract demonstrated anti-fungal effects against five species. Methanol root extracts exhibited anti-fungal activity only against *Alternaria* species, and methanol shoot extracts targeted *Pythium* and *Aspergillus* species. Overall, these results suggest robust anti-bacterial activity of the extracts against the tested bacteria and fungi (Kaur *et al*., 2006, 2008; Mohinder *et al*., 2010).

2.1.5.3 Hepatoprotective activity (J. Li *et al*., 2021)

The hepatoprotective property in the decoction of *H. candicans* roots was determined using carbon tetra chloride (CCl_4) as inducing agent. As per this study, the root extract showed significant hepatoprotective activity that the CCl_4 treated groups. Pathological

sections in pretreated groups exhibited reduced vacuole formation, neutrophil infiltration, and necrosis.

2.1.5.4 Other reported activities

Other activities that have been reported on this plant include: anti-oxidant (Coruh *et al*., 2007); anti-viral and immuostimulant (Webster *et al*., 2006); Leucoderma (B. Joshi & Tyagi, 2011); Psoriasis (Dewick, 2009).

2.2 Role of phytochemicals in vitiligo

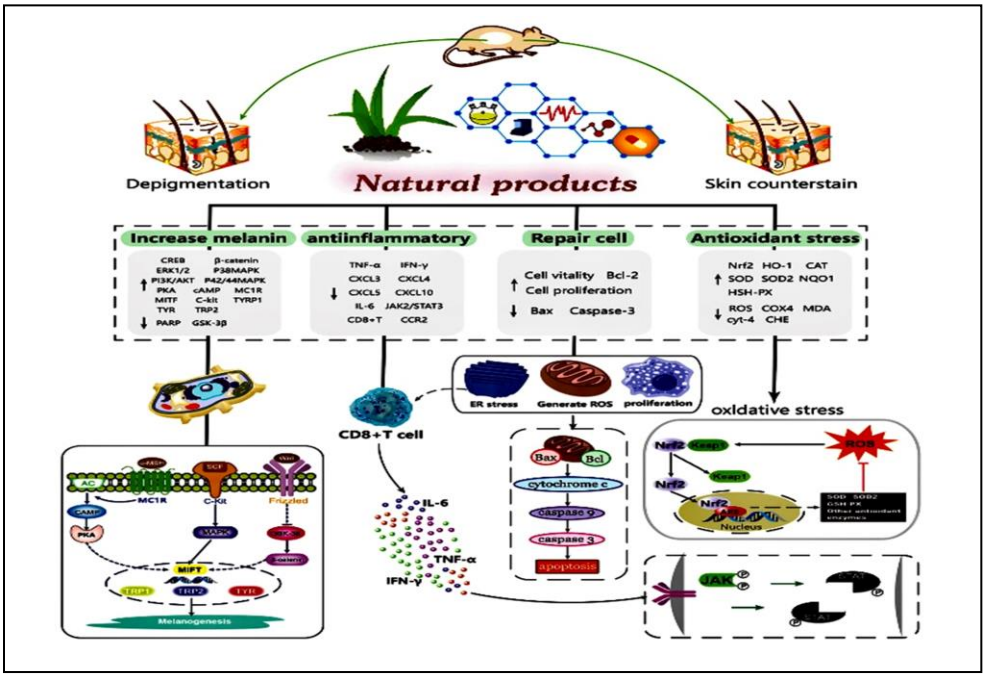


Figure 2: Natural products and vitiligo (Pang *et al*., 2021)

2.3 Role of anti-oxidants in vitiligo

Anti-oxidants, described as molecules with the ability to stabilize or neutralize free radicals, are integral to the complex enzymatic and non-enzymatic anti-oxidant systems evolved by humans. These systems work synergistically to protect cellular organs from stress-induced damage reactive nitrogen oxygen radicals (Halliwell, 1995). Anti-oxidants have multiple purposes such as destroying free radicals, supplying hydrogen or electrons, fragmenting down peroxides, eliminating the singlet oxygen, limiting enzymes,

amplifying the impacts of other products, and associating to metal ions. Rice-Evans and Diplock (1996) propose two main mechanisms: the chain-breaking mechanism, where primary anti-oxidants donate electrons to neutralize free radicals, and the removal of nitrogen-reactive molecule or oxygen-reactive molecule initiators by secondary anti-oxidants (Rice-Evans *et al*., 1996). Krinsky (1992) highlights their effects through electron donation, metal ion chelation, co-anti-oxidant collaboration, and gene expression regulation (Krinsky, 1992).

2.3.1 Disease conditions and oxidative stress

In the realm of biological systems, radicals, specifically hydroxyl, superoxide, and nitric oxide, frequently recognized to as reactive oxygen species (ROS) and reactive nitrogen species (RNS), harbor the potential to inflict harm upon DNA and disrupt the delicate Equilibrium amongst oxidants and anti-oxidants. The excessive generation of these radicals, triggered by factors like smoking, exposure to radiation, alcohol consumption, and contact with environmental toxins, is intricately linked to the onset and progression of chronic and degenerative medical conditions (Baiano & Del Nobile, 2016). Increasing the intake of exogenous anti-oxidants, particularly those derived from plant materials, demonstrated to mitigate oxidative stress-induced damage. Polyphenols, carotenoids, and vitamins, abundant in agricultural by-products, perform as quenchers of singlet oxygen, scavengers of free radicals, reducing agents, and ameliorating the impact of oxidative stress (Baiano & Del Nobile, 2016; Manach *et al*., 2004). Anti-oxidants, obtained either endogenously or exogenously, possess a crucial part in sustaining ideal cell functions, endogenous anti-oxidants are crucial for systemic health, conditions promoting oxidative stress may necessitate dietary anti-oxidants (Matés *et al*., 1999).

2.3.2 Biological effects of anti-oxidants from nature

Polyphenols and carotenoids, prominent natural anti-oxidants, display a varied array of physiological impacts, including anti-bacterial, anti-inflammatory, anti-aging, anti-viral, and anti-cancer properties (Li *et al*., 2014; Li *et al*., 2015; Wojtunik-Kulesza *et al*., 2016; Zhou *et al*., 2016). Anti-oxidant substances which require no catalysts are essential

components in this resistance structure (McCall, 2000). Dietary compounds enhancing the activity of endogenous anti-oxidants are also considered vital in the anti-oxidant classification. Recognizing the interplay between endogenous and dietary anti-oxidants provides insights into maintaining optimal cellular functions under varying physiological conditions.

2.3.3 Anti-oxidants classification

Three distinct types of anti-oxidants exist:

1. Primary anti-oxidants at the forefront comprise catalase, superoxide dismutase, glutathione reductase, and essential minerals such as Cu, Se, and Zn.
2. Secondary defense anti-oxidants, encompassing glutathione, albumin, vitamin C, vitamin E, flavonoids and carotenoids.
3. Tertiary defense anti-oxidants, constituting a diverse group of enzymes accountable for repairing damaged DNA, oxidized lipids, proteins, and peroxides. Illustrations include DNA repairing enzymes, lipase, protease, methionine sulfoxide reductase and transferases (Irshad & Chaudhuri, 2002).

2.3.4 Furanocoumarins as anti-oxidants

Furanocoumarins, natural chemicals found in various plants and fruits, have been recognized for their anti-oxidant properties and potential health benefits. These compounds have gained considerable research interest due to their capability to shield cells from oxidative impairment and combat free radicals stress (Hoult & Payá, 1996). Genus Heracleum was lately discovered to possess many furanocoumarins and their derivatives, which play fundamental roles in the therapeutics including oxidative strain (Ramazani *et al*., 2019). Many researchers have been steered on the furanocoumarins bearing plants and their role in the anti-oxidation including the plants such as: *Angelicae dahuricae* (Zheng *et al*., 2016), *Heracleum verticillatum, Heracleum sibiricu, Heracleum angustisectum* (Ozek *et al*., 2019), *Ferulago trifida* (Tavakoli *et al*., 2018), *Angelica officinalis* (Senol *et al*., 2011), *Ferulago subvelutina* (Gohari *et al*., 2013), *Heracleum*

sprengelianum (Karuppusamy & Muthuraja, 2011), *Heracleum nepalense* (Dash *et al*., 2005), *Heracleum persicum* (Coruh *et al*., 2007).

2.3.5 The anti-oxidant power of plants

Anti-oxidants from nature predominantly consist of phenolics, which can be found in various plant components (Asif, 2015). Recently, toxicological investigations into the application of synthetic anti-oxidants have revealed undesirable or negative effects. Some of the findings have prompted investigators to redirect their investigations toward uncovering natural sources exhibiting considerable anti-oxidant potential (Ramalakshmi *et al*., 2008). Plants produce diverse secondary metabolites to interact with their environment, including terpenes, alkaloids, and polyphenols.

Table 5: Catalog of plants frequently utilized as potential sources of anti-oxidants

Name of plants	Family	Plant part used	Reference
Aegle marmelos	Rutaceae	Fruit pulp	(Rajan *et al*., 2011)
Allium cepa	Amaryllidaceae	Bulb	(Mantawy *et al*., 2012)
Allium sativum	Alliaceae	Bulb	(Rahman *et al*., 2012)
Aloe vera	Xanthorrhoeaceae	Leaf	(Hassanpour, 2015)
Asparagus racemosus	Liliaceae	Shoot	(Karuna *et al*., 2018)
Azadirachta indica	Meliaceae	Leaf	(Deka *et al*., 2013)
Bacopa monniera	Plantaginaceae	Leaf	(Simpson *et al*., 2015)
Beta vulgaris.	Amaranthaceae	Root	(Pyo *et al*., 2004)
Camellia sinensis	Theaceae	Leaf	(Vishnoi *et al*., 2018)
Capsicum annuum	Solanaceae	Fruit	(Hervert-Hernandez *et al*., 2010)
Curcuma longa	Zingiberaceae	Rhizome	(Maizura *et al*., 2011; G. Singh *et al*., 2010)
Cuscuta reflexa	Convolvulaceae	Stem	(Perveen *et al*., 2013)
Daucus carota	Apiaceae	Root	(Shyamala & Jamuna, 2010)
Emblica officinalis	Euphorbiaceae	Fruit	(Bhattacharya *et al*., 1999)
Eucalyptus camaldulensis	Myrtaceae	Leaf	(Ashraf *et al*., 2015)
Eugenia caryophyllus	Myrtaceae	Fruit, Leave	(Teixeira *et al*., 2013)
Foeniculum vulgare	Apiaceae	Fruit oil	(Mohamad *et al*., 2011)
Geranium sanguineum	Geraniaceae	Aerial parts	(Nikolova *et al*., 2010)
Hedychium spicatum	Zingiberace		(Bhatt *et al*., 2013)
Lavandula angustifolia	Lamiaceae	Aerial parts	(Dobros *et al*., 2022)

Leea indica	Vitaceae	Leaves	(Reddy *et al*., 2012)
Mangifera indica	Anacardiaceae	Pulp	(Rocha Ribeiro *et al*., 2007)
Nardostachys jatammansi	Valerianaceae	Root	(S. K. Sharma & Singh, 2012)
Nelumbo nucifera	Nymphaeaceae	Plant, seed	(M. Zhu *et al*., 2017)
Ocimum sanctum	Lamiaceae	Whole plant, seed	(Chaudhary *et al*., 2020)
Ocimum basilicum	Lamiaceae	Leaf	(Fitsiou *et al*., 2016)
Origanu mvulgare	Lamiaceae	Whole plant	(Coccimiglio *et al*., 2016)
Phaseolus vulgaris	Fabaceae	Fruit	(Oomah *et al*., 2010)
Phyllanthu semblica	Euphorbiaceae	Fruit	(Poltanov *et al*., 2009)
Phyllanth usniruri	Euphorbiaceae	Fruit	(Rusmana *et al*., 2017)
Phyllanthus urinaria	Euphorbiaceae	Fruit	(Yong Liu & Li, 2020)
Picrorhiza kurrooa	Scrophulariaceae	Root	(Kant *et al*., 2013)
Piper nigrum	Piperaceae	Fruit	(Zhang *et al*., 2021)
Plantago asiatica	Plantaginaceae	Seed	(Dong *et al*., 2020)
Plumbago zeylanica	Plumbaginaceae	Flowers	(Beyene *et al*., 2020)
Polyalthia cerasoides	Annonaceae	Roots	(Tekuri *et al*., 2019)
Prunus domestica	Rosaceae	Fruit	(Kayano *et al*., 2002)
Punica granatum	Punicaceae	Seed, pericarp, rind	(Mutahar S *et al*., 2012)
Rubia cordifolia	Rubiaceae	Roots	(Humbare *et al*., 2022)
Salvia officinalis	Lamiaceae	Aerial parts	(Kontogianni *et al*., 2013)
Santalum album	Santalaceae	Heartwood, bark	(Mohankumar *et al*., 2019)
Solanum nigrum	Solanaceae	Leaf	(Campisi *et al*., 2019)
Solanum tuberosum	Solanaceae	Tuber	(J. Kim *et al*., 2019)
Sphaeranthus indicus	Asteraceae	Flowers	(Tandon & Gupta, 2020)
Strigaoro banchoides	Scrophulariaceae	Whole plant	(Badami *et al*., 2003)
Taxus baccata	Taxaceae	Leaves, bark	(Bekhouche *et al*., 2022)
Terminalia bellarica	Combretaceae	Fruit	(A. Gupta *et al*., 2021)
Thymus vulgaris	Lamiaceae	Whole plant	(Lemos *et al*., 2017)
Trigonellafoenum-graecum	Fabaceae	Leaf, seed	(Akbari *et al*., 2019)
Valeriana jatamansi	Valerianaceae	Roots	(Jugran *et al*., 2021)
Withania somnifera	Solanaceae	Root, leaf and seed	(Dhanani *et al*., 2017)
Zingiber officinale	Zingiberaceae	Rhizome	(Tohma *et al*., 2017)

CHAPTER 3 PLAN OF WORK

Plan of Work

The central objective of the collated research endeavors was the development of a topical formulation containing a potent anti-vitiligo agent. This goal was propelled by the acknowledged assertions highlighting the potential efficacy of the anti-vitiligo properties in light of *Heracleum candicans*, this research is expected to bring a ray of hope in vitiligo treatment by a dosage form incorporated with the herbal extract from aerial part of *H. candicans* in a suitable formulation for anti-vitiligo activity.

3.1 The aim of the research work is

Development, evaluation and characterization of novel anti-vitiligo ointment from Himalayan *Heracleum candicans* Wall. ex DC.

The objectives are as follows:

- ✓ To standardize and extract the aerial part of *H. candicans.*
- ✓ To evaluate the extract of aerial part of *H. candicans* for anti-vitiligo activities.
- ✓ To formulate a suitable topical formulation (ointment) from aerial part of *H. candicans*.
- ✓ To evaluate and characterize the topical formulation (ointment) from aerial part of *H. candicans.*
- ✓ To perform anti-vitiligo activity of topical formulation (ointment).
- ✓ To perform anti-oxidant activity of topical formulation (ointment).

3.2 Flow chart of plan of work

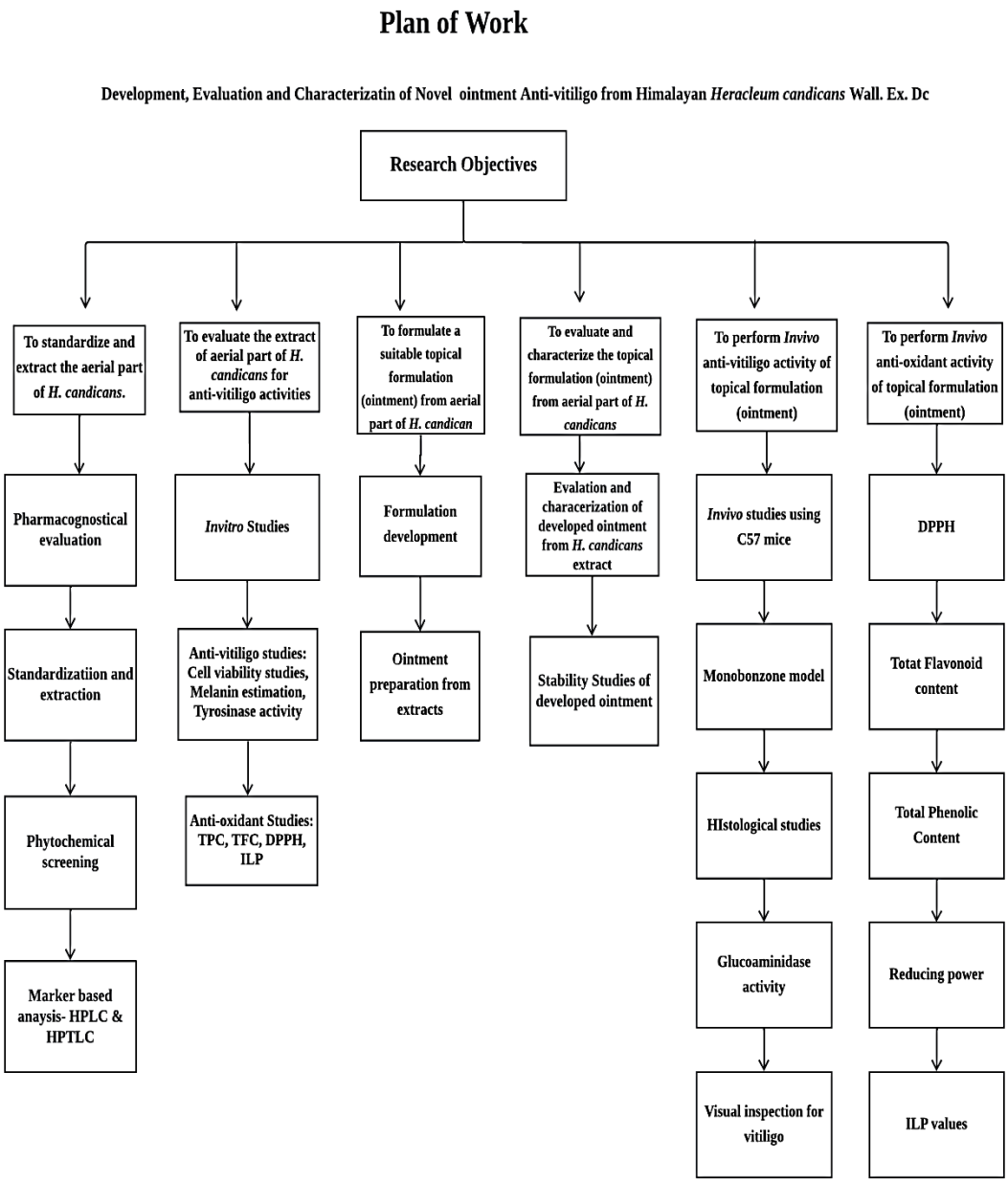


Figure 3: Plan of work

CHAPTER 4 COLLECTION, STANDARDIZATION AND EXTRACTION

The search for medicines for various disease conditions from natural resources has been performed since ancient times. Various knowledge exchange, memorials, and written documentation are evidence for this. Around two-thirds of novel medications were directly or indirectly developed from plants. Hence, comprehensive studies on phytoconstituents from traditional medicines using phytochemical, pharmacological, and analytical methods are the need of the hour (Nissar *et al*., 2021). The standardization of crude drugs is of paramount importance that begins with the collection of botanical materials and ends with their packaging and usage as medicine. Microscopy, macroscopy, physicochemical characteristics, extractive values, fluorescence analysis, and heavy metal analysis are utilized to set pharmacognostical standards. These characteristics, in turn, can serve to ensure the drug's quality (Khandelwal, 2008). However, because the taxonomy of this genus is challenging, botanists require more and better samples for authentication (Kaul, 1989). Most of the Heracleum species are utilized as medicinal plants, herbs, or spices. Several traditional uses exist for *H. persicum, H. sphondylium*, and *H. candicans* in particular. They are used to treat epilepsy, flatulence, stomachache, used as analgesic, carminatives, digestives, antiseptics, and anticonvulsants (Bahadori *et al*., 2016). Many specialized metabolites such as coumarins, flavonoids and lignans have been isolated and identified from the genus Heracleum (Dadjo *et al*., 2015). This genus has the potential to develop new coumarin derivatives. These compounds exhibit anti-Alzheimer, anti-neurodegenerative, and anti-oxidant, anti-cancer, anti-diabetic, anti-bacterial, anti-viral, and anti-inflammatory effects. Researchers have studied a few of the biological effects of Heracleum including anti-bacterial, anti-proliferative, and anti-inflammatory (Walasek *et al*., 2015). The species *H. candicans* is of substantial commercial importance being a major source of xanthotoxin, which are widely used in the treatment of leucoderma as a component of sun-tan lotion. Xanthotoxin has been isolated from many Himalayan and

non-Himalayan plant species of Heracleum for instance, *H. mantegazzianum* and *H. sphondylium, H. yunngningense, H. rapula, H.lanatum, H. persicum, H. sibiricum* and some other *Apiaceae* species like *Ammi majus* and *Angelica japonica.* Among the Himalayan species, *H. candicans* has maximum percentage of xanthotoxin (1.5%) followed by *H. cachemiricum* (0.05%), *H. canescens* (0.005%) and *H. pinnatum* (0.005%) (Bahadori *et al*., 2016; Banerjee *et al*., 1979; Nayar, 1996). To the best of our knowledge detailed pharmacognostic and standardization studies are not available for this plant. Therefore, this study was carried out to establish important pharmacognostic parameters of this plant that could be beneficial in the identification of this plant. The results of preliminary phytochemical analysis of various *H. candicans* extracts revealed the presence of carbohydrates, tannins, phenols, flavonoids, phytosterols, coumarins, proteins, saponins, diterpenes, cardiac glycosides, fats and oils. However, alkaloids and anthraquinone glycosides were found to be absent in all the extracts. The pharmacognostic studies of *H. candicans* revealed the presence of prismatic calcium oxalate crystals, anomocytic stomata, and simple starch grains along with two types of covering trichomes which were more numerous in the lower epidermis of the leaves. It also revealed the presence of spiral and reticulated xylem vessels (lignified) along with lignified fibers which can assist in proper identification of this less explored drug. The estimation of various physicochemical constants such as extractive values, ash values, loss on drying, pH value, swelling index, foaming index, heavy metal analysis, fluorescence analysis and TLC values can be helpful in determining various quality control standards for the crude drug. Additionally, the absence of heavy metals in the plant also depicts the better environment conditions of the study area for the cultivation of the medicinal plants. Pharmacognostic study of this less explored plant will be useful in the correct identification of this plant for future references and preparation of monographs.

4.1 Materials and Methods

4.1.1 Collection and authentication of plant material

Aerial parts of *H. candicans* were collected in the month of July-August, from an altitude of 2850-2900 m above the sea level, from the foot hills of Betaab Valley, Pahalgam, District Anantnag, and Ferozpora, Tangmarg, District Baramulla, Jammu and Kashmir, India. Herbarium specimen was prepared and the plant was authenticated and identified by the Centre for Biodiversity and Taxonomy, Department of Botany, University of Kashmir with voucher specimen number 2118-KASH & 2847-KASH.

4.1.2 Macroscopic study

Macroscopic evaluation is an important parameter to establish the identity of the crude drugs. Macroscopic characters of a drug include its visual appearance to the naked eye. Thus, detailed morphological study of the characters could be beneficial in distinguishing them. The macroscopic evaluation of *H. candicans* was done as per the method described earlier (Panahi *et al*., 2015).

4.1.3 Microscopic study

Various cellular structures like epidermal trichomes, calcium oxalate crystals, starch granules, lignified tissues were carefully examined for their size, shape and histochemistry was also performed by various staining reagents (World Health Organization., 1998). The dried aerial parts were powdered and the microscopic characters were examined by using various staining reagents (Thitikornpong *et al*., 2011). The characteristic structures of cells and its components were observed followed by capturing photomicrographs. Other staining reagents likeruthenium red, toluidine blue, were also used for microscopy (Parker *et al*., 1982).

4.1.4 Physiochemical evaluation

The physicochemical parameters like extractive values, ash values and foaming index was evaluated as per the prescribed method (Lohar, 2008; World Health Organization., 2011).

4.1.5 Determination of pH

The pH of aqueous solution (1% and 10%) of the powdered drug was carried out using a calibrated glass electrode.

4.1.6 Fluorescence analysis and powder drug reaction with various reagents

Several plant materials exhibit fluorescence on exposure to UV light and this property is utilized in plant identification. The fluorescence study of the samples was carried out in daylight and UV light (254 nm and 366 nm) both. Likewise, after treatments with different chemical reagents for instance nitric acid, sodium hydroxide, iodine, acetic acid, picric acid, hydrochloric acid, ferric chloride, etc. were also analyzed (Chase Jr & Pratt, 1949; Kokoski *et al*., 2006). Analysis of powdered plant material with various chemical reagents was also conducted.

4.1.7 Preliminary phytochemical investigation

Plant extracts obtained were then exposed to qualitative phytochemical screening to identify the presence of various primary and secondary metabolites like glycoside, alkaloids, flavonoids, tannins, sterols and steroids, terpenes and terpenoids, phenolic compounds, coumarins, mucilage, resins, carbohydrates, protein, amino acids, saponins, and fats and oils, etc. (Harborne, 1998).

4.1.8 Thin Layer Chromatography

The samples of plant extracts were applied on silica gel G TLC plates with the help of capillary tubes. TLC chamber was then utilized for the development of these plates using appropriate mobile phase. The mobile phase was optimized based on the hit and trial method. Chromatographic rectangular glass chamber (16.5cm x 29.5cm) and pre-saturated TLC chamber were used for this purpose. The plates were exposed to iodine vapors after completion of air drying. Retention factor (R*f*) was estimated individually for all the samples (Biradar, 2013).

4.1.9 Heavy metal analysis by ICP-OES Instrument

The heavy metals analysis of powdered plant material is an important tool to evaluate the quality of crude drugs. Heavy metal evaluation for the aerial parts of *H. candicans* was done by ICP-OES method at Avon Food Lab (Pvt) Ltd, Delhi (NABL accredited). Four

main heavy toxic metals Cd, As, Pb and Hg were evaluated for their presence in the aerial parts of *H. candicans.*

4.1.10 Extract preparation using Soxhlet apparatus

After collection and authentication, aerial plant material were dried in shade and powdered. The powdered material was sieved by 40 mesh sieves, accurately weighed and used for extraction. The successive extraction was done by different solvents like petroleum ether, chloroform and methanol in Soxhlet apparatus on the other hand; decoction method was used for aqueous extraction. The extracts were concentrated under reduced pressure through rotary evaporator and dried. The resulting extract was weighed and stored in airtight containers (amber coloured) at temperature between 5-7°C till further use (Evans, 2009).

4.2 Results of the study

4.2.1 Macroscopic evaluation

The leaves of the plant are broad ovate, around 3-9 cm in length and 3-6 cm wide, with lower surface of leaves having papery texture. The stem is elongated, yellowish green in color, around 1-2 cm in diameter and 10-18 cm long (Figure 4). The powder of the aerial parts was yellowish green in color with pleasant odour and astringent taste.

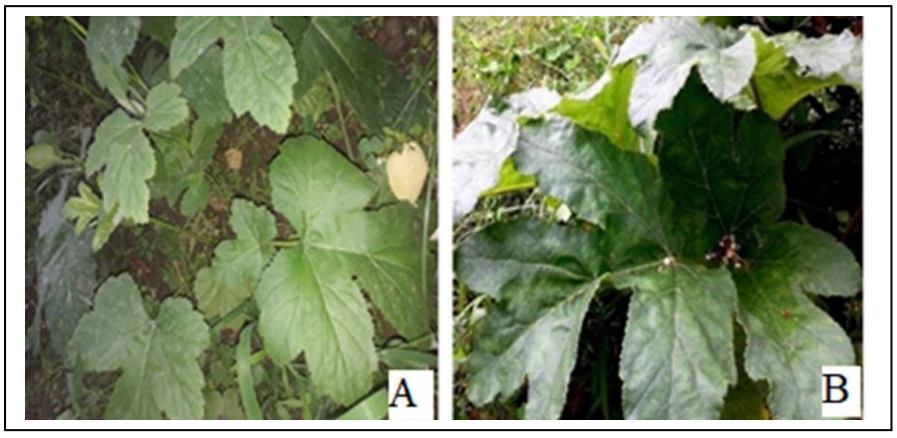


Figure 4: Original images of *Heracleum candicans* in its habitat (A-B)

The macroscopic study of the aerial parts of *H. candicans* revealed the presence of white-felted underside of the large pinnately lobed leaves with characteristic shape and faint

odour. Lower and basal leaves were pinnate; 2-3 pinnate pairs, ovate-oblong, 4-13 cm long, 3-6 cm wide, densely white tomentose, abaxially silvery, margins serrate with obtuse apex. Flowers, white; the outer petals of the flowers are larger, bilobed. Stem is solitary, branched. White petals, minute calyx teeth, radiant outer flowers of umbels, fruit, obovoid, around 6-12 mm long, 4-7 mm wide, glabrous when mature (Figure 5).

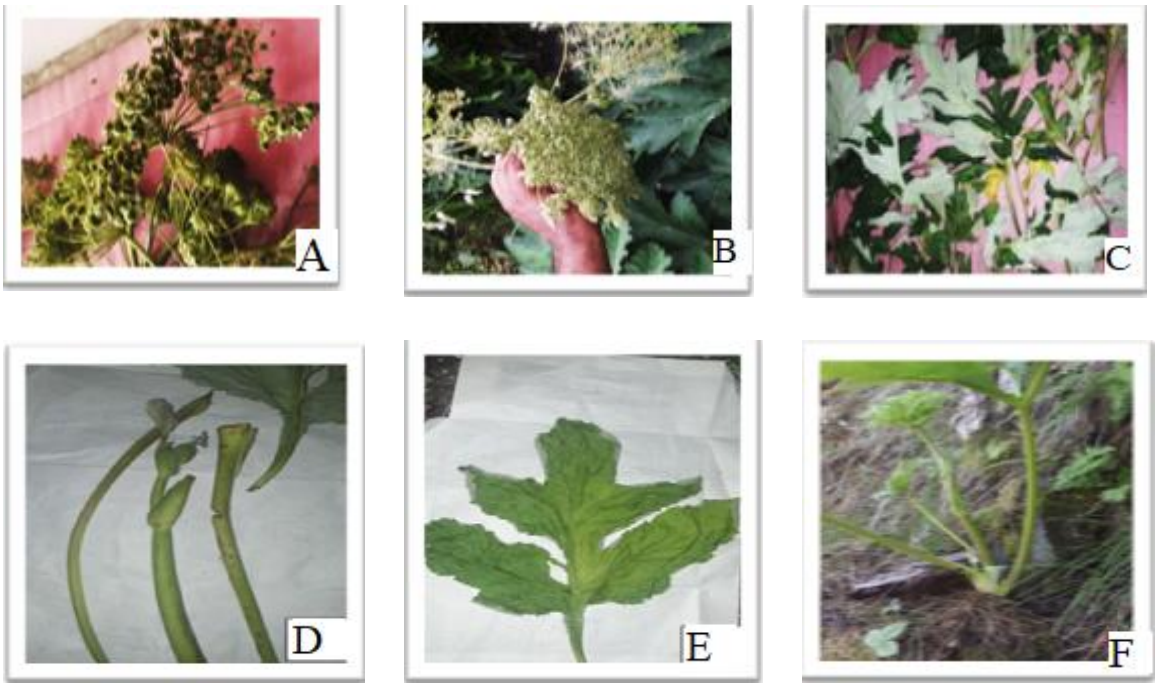


Figure 5: Macroscopic study of aerial parts of *H. candicans (*A, B= Dried Seeds; C= Dried aerial leaves and stem; D= Stem*;* E= Surface view of Leaf; F= Plant in natural habitat).

4.2.2 Microscopic evaluation

The diagnostic features of powder microscopy of aerial part of *H. candicans* in surface view revealed lamina fragments. Upper epidermis was formed by wavy walled polygonal cells, irregularly thickened and beaded. There was more elongation in the cells over the vein's region; underlying small palisade cells were closely packed. The detailed microscopic studies are depicted in figure 6.

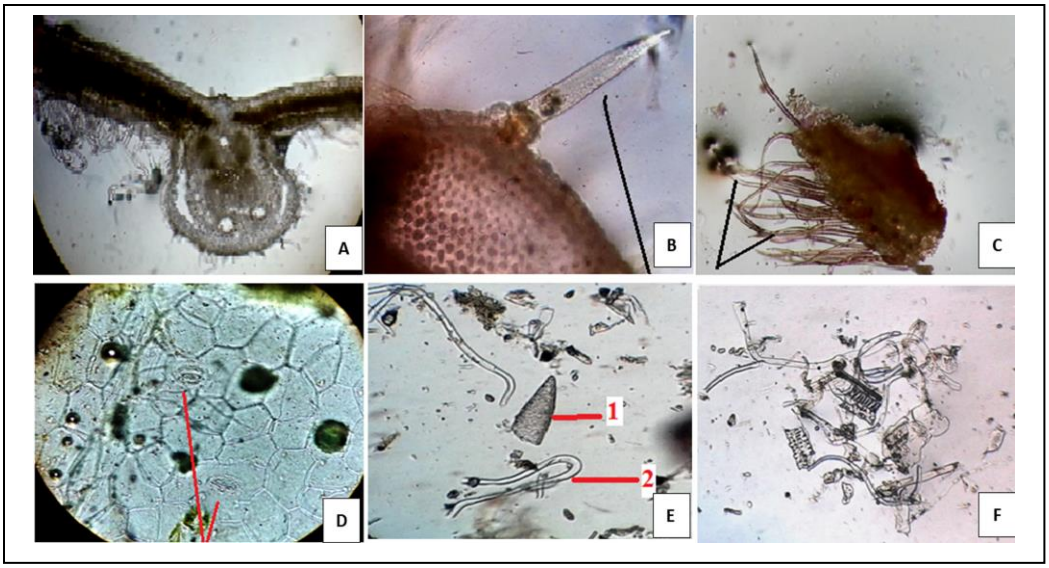


Figure 6: Microscopic study of *H. candicans* aerial part (A: T.S. of leaf; B: Unicellular covering trichome; C: Presence of abundant covering trichomes in the lower leaf surface; D: Anomocytic stomata in aerial part; E: Covering trichomes at upper and lower surface of leaf; F: Prismatic Ca. oxalate crystals and spiral xylem vessels in aerial part).

The cytological structures of lower epidermis were smaller than upper epidermis; numerous small anomocytic stomata were also found; occasionally lignified cicatrices and abundance of covering trichomes were also found. The abundant crystals of prismatic calcium oxalate were found in powder of aerial parts of *H. candicans*. They were variable in size and some of them were moderately large. From the veins and rachis, fibres which are lignified and groups of vascular tissue were also seen. Thickened walls of fibres; few pits and the vessels were also lignified which were thickened reticulately or annularly. Lignified xylem vessels were found to be of two types i.e., reticulated and spiral shaped in the powder of *H. candicans.* Simple starch grains were also seen in the powder microscopy particularly in the aerial part. Stomata that were seen in the parenchymatous tissue of aerial powder were of anomocytic type. The detailed microscopic characters for the aerial parts are presented in figure 7.

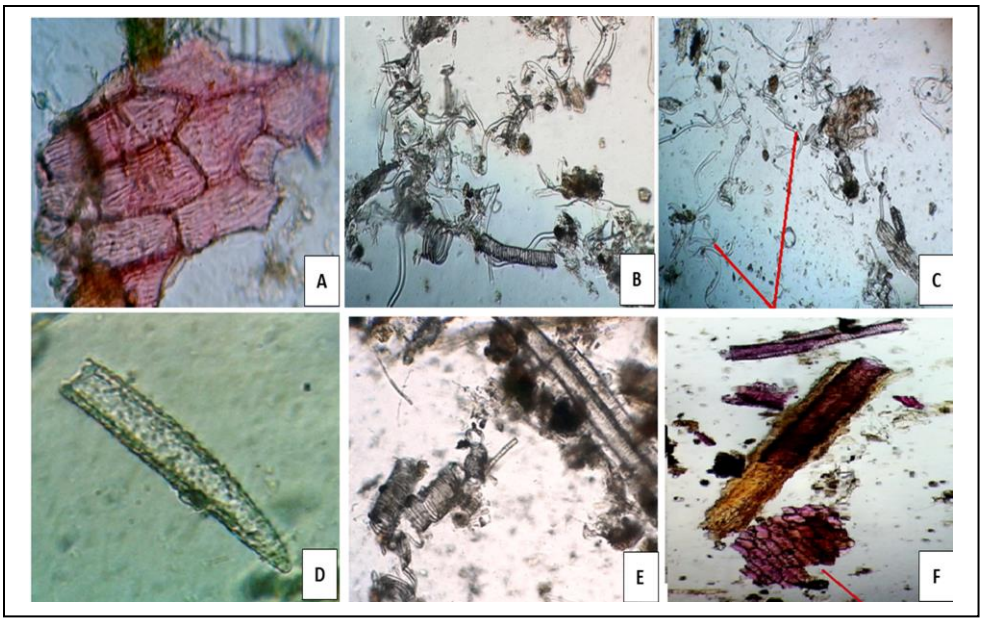


Figure 7: Powder microscopic study of *H. candicans* aerial part (A: Lignified pitted parenchyma in aerial part; B: Aerial part showing spiral & reticulated xylem vessels; C: Abundant covering trichomes in aerial part; D: Aerial powder showing a covering trichome under 40x; E: Reticulated xylem vessels in aerial part; E: Lignified pitted parenchyma in aerial part).

4.2.3 Physicochemical parameters

While carrying out the cold extraction of aerial part, the maximum cold extractive value was found in methanol extract (17.20% w/w), followed by aqueous extract (14.25% w/w) chloroform (2.95% w/w), ethyl acetate (1.56% w/w) and petroleum ether (1.05% w/w). Hot extractive values of methanol, aqueous, chloroform, ethyl acetate and petroleum ether extracts of aerial part were 20.30% w/w, 16.65% w/w, 4.45% w/w, 2.15% w/w and 2.80% w/w, respectively. Successive extractive value in aerial part was maximum in methanol (22.65% w/w) followed by aqueous (18.07% w/w), chloroform (5.15% w/w), ethyl acetate (3.25% w/w) and petroleum ether (2.85% w/w). The details are presented in figure 8.

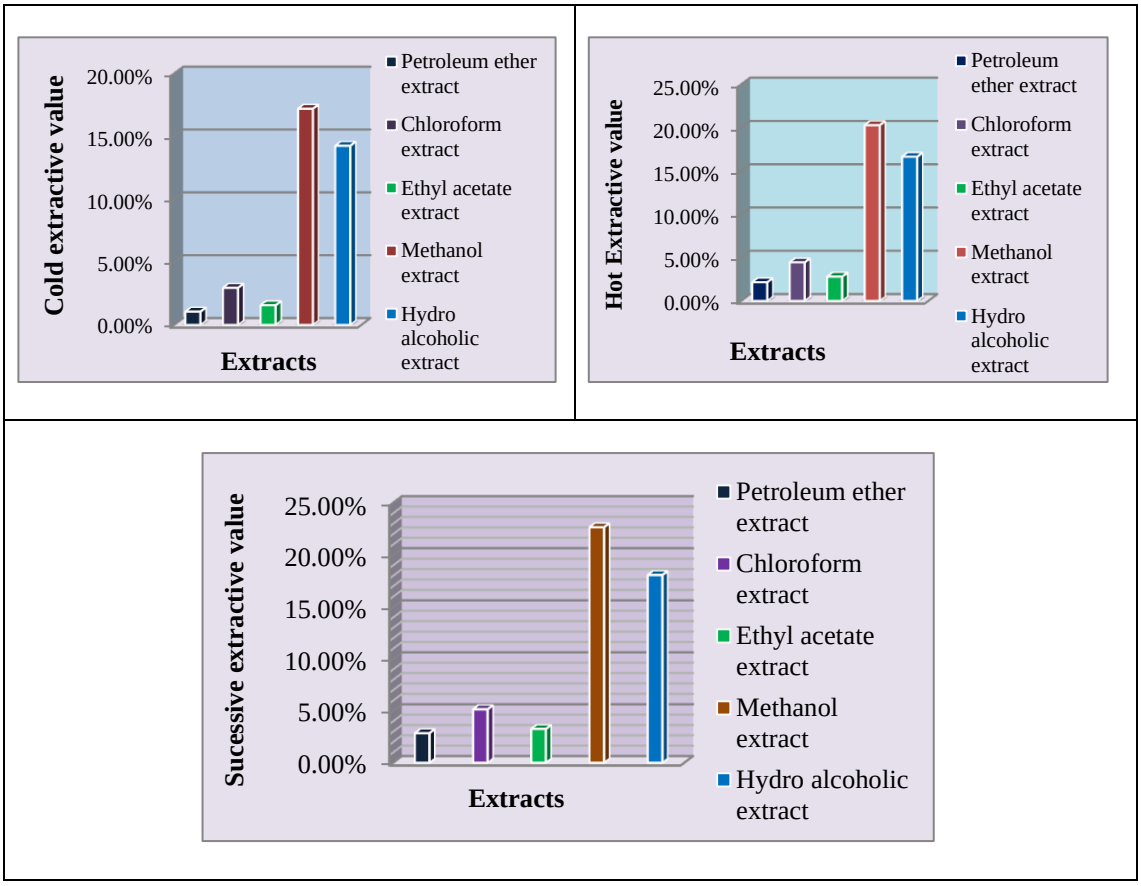


Figure 8: Cold, hot and successive extractive values of *H. candicans* aerial parts

The pH value of 1% and 10% solution of aerial parts in distilled water were found to be 6.24 and 6, respectively representing presence of different constituents of acidic nature in this plant. The active chemical constituents' percentage in crude drugs is calculated on air dried basis. The loss on drying of dry powder was 11.73%. The foaming index was found to be less than 100 for aerial powder of *H. candicans*. The total ash value which represents the inorganic and non-physiological matter such as silica occurring naturally in the drug or adhering to it was found to be 7.8%. Swelling index for the aerial plant powder was found out to be 2.8, while as the foreign material present in the powdered drug was found to be 0.065. The details pertaining to physicochemical analysis are presented at table 6.

Table 6: Qualitative phytochemical studies of aerial parts of *H. candicans*

Parameter	Result
Loss of drying (% w/w)	11.73± 0.50
Total ash value (%w/w)	7.8± 0.21
Water soluble ash value (% w/w)	2.66± 0.36
Acid insoluble ash value (% w/w)	3.2 ± 0.89
Foaming index	<100
Swelling index	2.8± 0.18
pH (1%)	6.24± 0.15
pH (10%)	6.0± 0.79
Foreign matter (%)	0.065

4.2.4 Fluorescence analysis

Fluorescence analysis was performed with various reagents to observe the difference in the physical characteristics of the crude drugs. The fluorescence analysis results of this plant are presented in table 7

Table 7: Fluorescence analysis of powdered drug of aerial parts*

Drug Treatment	Visible light	UV 254 nm	UV 360 nm
Powder drug as such	Water grey	Espresso	Black
Dist. Water	Brown	Cherry	Brown
conc. HCl	Brown	Blackish brown	Black
Dil. HCl (10%)	Light brown	Espresso	Black
Conc. H_2SO_4	Blackish brown	Brown	Black
Dil.H_2SO_4 (10%)	Steel grey	Cherry	Light brown
Nitric acid	Buff	Brown	Black
Dil. HNO_3 (10%)	Brown	Espresso	Black
10% NaOH	Brown	Brown	Brown
Picric acid	Dark green	Brown	Black
Iodine solution	Oxford blue	Brown	Brownish black
Methanol	Brownish black	Espresso	Black
Ethanol	Blackish brown	Brown	Black
Acetic acid	Espresso	Brown	Brownish black
Chloroform	Sugar creek	Espresso	Black
Petroleum ether	Sugar creek	Brown	Black
Ferric chloride	A.D grey	Espresso	Black
Ammonia solution	Sugar creek	Brown	Brown

*Compared with Kansai Nerolac synthetic Japan, S-2014.

4.2.5 Preliminary phytochemical screening

The preliminary phytochemical screening was done to identify the constituents of the plant and the results have been tabulated in table 8 which indicated the presence of various phytoconstituents like carbohydrates, alkaloids, flavonoids, polyphenolic compounds, tannins and terpenoids.

Table 8: Phytochemical screening of powdered drug

Test	Inference	Methanolic Extract	Hydroalcoholic extract	Ethyl acetate extract	Chloroform extract	Petroleum ether extract
CARBOHYDRATES						
Molisch's test	Violet ring	+	+	+	-	-
Fehling's test	Brick red ppt.	+	+	+	-	-
Benedict's test	Orange red ppt.	+	+	-	-	-
TANNINS						
5% $FeCl_3$ test	Yellow color	+	+	+	+	+
Lead acetate test	White ppt	+	+	+	+	-
Gelatin test	White ppt	+	+	+	-	-
FLAVONOIDS						
Shinoda test	Pink colour	+	+	+	+	+
Alkali reagent test	Intense yellow color which becomes colorless on addition of dil. acid	+	+	+	+	-
Lead acetate test	yellow color ppt.	+	+	+	+	+
PHENOLS						
1% $FeCl_3$	Bluish color	+	+	+	+	-
PHYTOSTEROLS						
Salkowski test	Golden yellow ring	+	+	+	-	-
Liebermann's test	Brown ring at junction	+	+	-	-	-
SAPONINS						
Foam test	Foaming	+	+	+	+	+
Froth test	Frothing	+	+	+	+	+

DITERPENES						
Copper acetate test	Emerald green color	+	+	+	-	+
FATS AND OILS						
Filter paper test or Stain test	Permanent stain on filter paper	+	+	+	+	+
COUMARINS						
Filter Paper test under UV light	Yellowish green Fluorescence	+	-	+	+	+
CARDIAC GLYCOSIDES						
Keller Kiliani test	Brown ring at junction	+	+	+	-	-
Legal test	Pink color	+	+	-	-	-
ALKALOIDS						
Mayer's test	Cream ppt.	-	-	-	-	-
Hager's test	Yellow ppt.	-	-	-	-	-
Dragendorff's test	Orange ppt.	+	+	-	-	-
Wagner's test	Reddish brown ppt.	-	-	-	-	-
ANTHRAQUINONE GLYCOSIDES						
Borntrager's test	Pink color	-	-	-	-	-

Similarly, analysis of the powdered drug with various chemical reagents was conducted and the results of analysis are presented in table 9.

Table 9: Analysis of powdered drug of *H. candicans* (aerial part) with various chemical reagents

Reagents used	Observation
Dist. Water	Buff colour
Conc. HCl	Golden Fleece
Sulphuric acid	Burnt brick
Nitric acid	Marengo
10% NaOH	El. Greco Bronze (brownish)
Picric acid	Ra. Gold
Iodine solution	Espresso
Methanol	Tata mimosa
Ethanol	Dark lime bright
Acetic acid	Water grey
Chloroform	Golden Fleece
Pet. Ether	Light Golden Fleece
Ferric chloride	Brownish gold
Ammonia solution	Green

4.2.6 Thin layer chromatography

After preliminary screening, an attempt was made to separate the individual chemical constituents from various extract was carried out by thin layer chromatography method. The details of optimized mobile phases are given in table 10.

Table 10: Thin layer chromatography various extracts of *H. candicans* in different mobile phase ratio

Extract	Optimized mobile phase
Petroleum ether extract	Toluene: Ethyl acetate (9.5: 0.5)
Chloroform extract	Ethyl acetate: Formic acid: GAA (10:1:1)
Ethyl acetate extract	$CHCl_3$: Ethyl acetate: CH_3OH: Glacial Acetic Acid (7:1:3:0.5)
Methanol extract of leaves	Chloroform: Methanol (8: 2)
Hydroalcoholic extract	Ethyl acetate: Formic Acid: Glacial Acetic Acid: Water (100:11:11:26)

TLC analysis clearly revealed separation of substantial phytoconstituents in almost every extract as presented in figure 9.

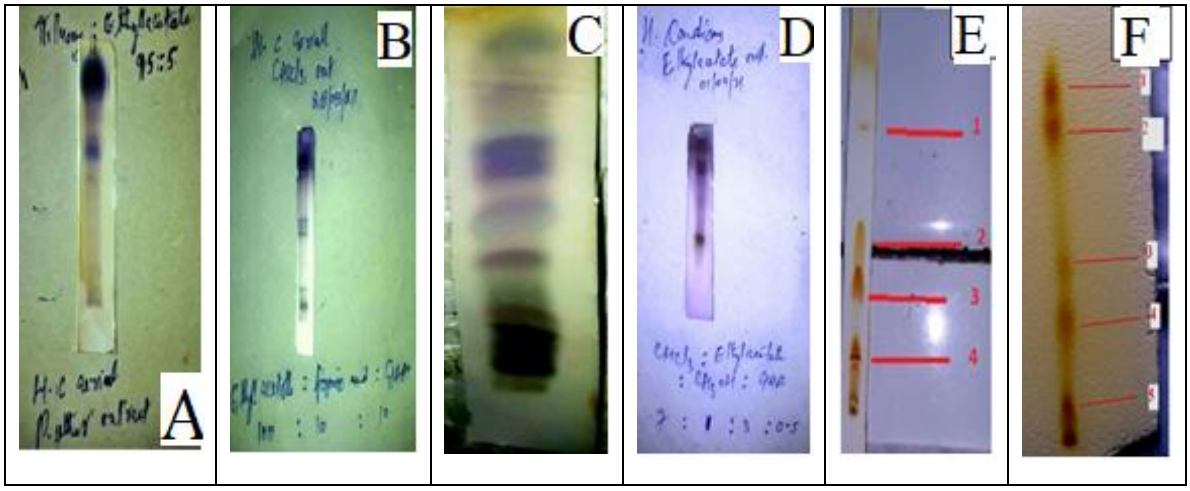


Figure 9: TLC chromatography of different extracts of *H.candicans* A. Petroleum ether extract (Toluene: Ethyl acetate 9.5: 0.5); B, C Chloroform extract (Ethyl acetate: Formic acid: GAA 10:1:1); D, Ethyl acetate extract ($CHCl_3$: Ethyl acetate: CH_3OH:Glacial Acetic Acid7:1:3:0.5); E.; Methanolic extract ($CHCl_3$:CH_3OH 8:2); F. Hydroalcoholic extract (Ethyl acetate: Formic Acid: Glacial Acetic Acid: Water (100 : 11: 11: 26).

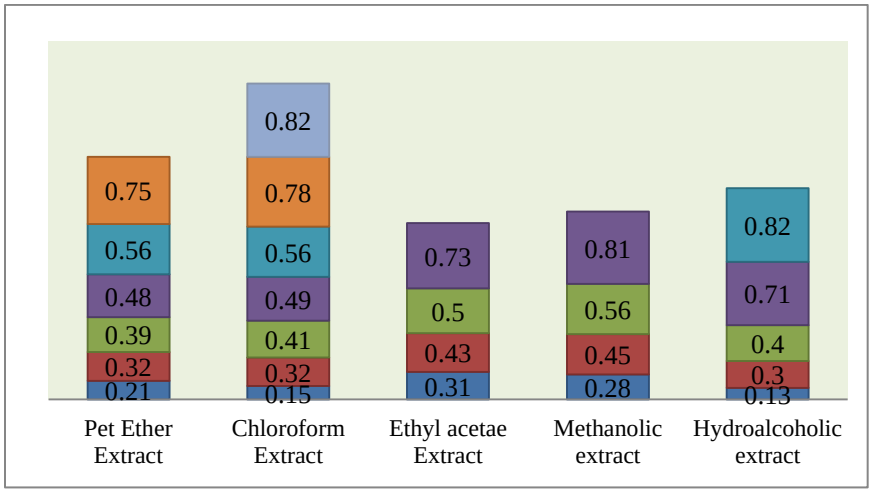


Figure 10: Number of phytoconstituents separated from various extracts through TLC with their R*f* values (Petroleum ether extract; Chloroform extract; Ethyl acetate extract; Methanolic extract; Hydroalcoholic extract of aerial parts).

The results of TLC studies showed that highest separation was occurred in the chloroform extract (7 spots at R*f* values 0.82, 0.78, 0.56, 0.49, 0.41, 0.32, 0.15), petroleum ether extract of leaf (6 spots at R*f* values 0.75; 0.56; 0.48; 0.39; 0.32 and 0.21). Followed by this, the aqueous extract of leaves also exhibited the presence of 5 spots at R*f* values 0.67, 0.53, 0.41, 0.39, 0.34 and 0.82, 0.71, 0.41, 0.30 and 0.13, respectively. Methanolic leaf extract showed presence of 4 spots. The details of R*f* values are presented in figure 10.

4.2.7 Heavy metal analysis

Heavy metal analysis is an important tool to check the quality of crude drugs. Four main heavy toxic metals Cd, As, Pb and Hg were evaluated for their presence in the aerial parts of *Heracleum candicans* and it was found that all these toxic metals were absent in the plant. This may be due to the geographical condition of the Himalayan region. The details of heavy metal analysis are present in table 11.

Table 11: Heavy metal residue of powdered drug

S. No.	Test Parameters	Result	MDL*
1	Cadmium (Cd)	Not detected	0.10 mg/kg
2	Lead (Pb)	Not detected	0.10 mg/kg
3	Arsenic (As)	Not detected	0.10 mg/kg
4	Mercury (Hg)	Not detected	0.100 g/kg

*MDL = Method Detection Limit; mg/kg = ppm

4.2.8 Microbial load of powdered drug

The results of the microbial load of powdered drug of *H. candicans* imply that the powdered sample is free from microbial contamination as shown in table 12**.**

Table 12: Microbial load of powdered drug

Parameter	Results (cfu/g)	Test Method
Total Plate Count	$1.1x10^2$	IS:5402:2012 (2018)
Yeast and Mold Count	<10	IS:5403:1999 (2018)

4.2.9 Results of mineral analysis using iCAP RQ (ICP-MS)

The results of the mineral analysis of the powder of aerial parts indicate that it is free from heavy metals and hence safe for medicinal use.

Table 13: Results of mineral analysis of extract

Name of element	Concentration average (ppm)
Sodium ^{23}Na (KED)	0.353
Magnesium ^{24}Mg (KED)	7.620
Potassium ^{39}K (KED)	107.063
Calcium ^{44}Ca (KED)	31.323
Chromium ^{52}Cr (KED)	0.037
Iron ^{57}Fe (KED)	2.717
Copper ^{63}Cu (KED)	0.034
Zinc ^{66}Zn (KED)	0.172
Cadmium ^{111}Cd (KED)	0.000
Lead ^{208}Pb (KED)	0.004

4.3 Discussion

Physico-chemical parameters are also vital for the standardization and quality control of herbal drugs which included foreign matter analysis, loss on drying, ash content, pH, swelling index, foaming index etc. Herbal materials should be devoid of any kind of contamination, so foreign matter analysis of powdered drugs can be considered as an important parameter in order to check the purity of herbal drugs (Organization, 1998). The present work demonstrates the pharmacognostic, physicochemical and preliminary phytochemical evaluation of aerial parts of *H. candicans*, which will help in appropriate identification of this plant for future investigation. The pharmacognostical study is a primary and reliable criterion in the identification and assessment of quality and purity of crude drugs. The macroscopic study of the aerial parts revealed the presence of white-felted underside of the large pinnately lobed leaves with characteristic shape and faint odour. Pinnate basal and lower leaves which were in 2-3 pairs, ovate-oblong, 4-13 cm long, 3-6 cm wide, pinnatified, silvery abaxially, apex obtuse, white tomentose, margins

serrate. Sessile reduced upper leaves, three lobed on expanded sheaths. Peduncles 15-28 cm long, bracts 1-3, linear, pubescent; caducous; rays 15-30, unequal, 3-10 cm long; umbellules 20-25 flowered. Flowers are white - the outer petals of the flowers are larger, bilobed. Stem is solitary, branched. Minute calyx teeth along with white petals, outer flowers of umbels radiant, Fruit obovoid, 6-12 mm long, 4-7 mm wide, glabrous when mature.

The diagnostic features of powder microscopy of aerial part of *H. candicans* in surface view revealed the presence of fragments of lamina. Upper epidermis is made up of polygonal cells which have slightly wavy walls, beaded and irregularly thickened. In areas over the veins, cells were more elongated. Numerous unicellular and non-lignified covering trichomes with elongated cells were present.

The abundant prismatic calcium oxalate crystals of variable size were found in powder of aerial parts of *H. candicans*. Some other diagnostic cellular structures include lignified fibres; few pits and the reticulate and spiral vessels. Stomata that were seen in the parenchymatous tissue of aerial powder were of anomocytic type.

Extractive value plays a vital role in the evaluation of crude drugs and gives an idea about the active constituents present in the drug. It is also useful for the estimation of specific constituents, soluble in that particular solvent used for extraction. Extractive values are primarily useful for the determination of exhausted or adulterated drugs (Thomas *et al*., 2008). High alcohol soluble and water-soluble extractive values (cold, hot and successive) in aerial parts revealed that the plant mainly contains polar substances. The pH value of 1% and 10% solution of leaf in distilled water were found to be 6.24 and 6.00, respectively representing the presence of acidic constituents in the plant.

Preliminary phytochemical screening revealed the presence of various phytoconstituents like carbohydrates, alkaloids, flavonoids, tannins, phenolic compounds and terpenoids. Moreover, the method for TLC of the plant was also developed and it was found that almost all the extracts utilized in the TLC studies showed substantial amount of phytoconstituents. The phytochemical screening of *H. afghanicum* has been found to

show phenolic substances (flavonoids and total phenols), sugars, resins, and sterols, but no alkaloids or saponins (Amini *et al*., 2017). In a similar manner, the qualitative analysis of stem, leaves, flowers, and fruits of *H. persicum* showed the presence of various phytochemicals like flavonoids, tannins, fixed oils and steroids while the carbohydrates, alkaloids, anthraquinones, cyanogenetics and saponins were not found in samples (Yazdinezhad *et al*., 2016). The alkaloid content of *H. candicans* extracts might have anti-convulsant and cytotoxic effects as reported for *H. persicum* (Afrisham *et al*., 2015). Moreover, the method for TLC of the plant was also developed and it was found that almost all the extracts utilized in the TLC studies showed substantial amount of phytoconstituents.

CHAPTER 5 MARKER BASED ANALYSIS OF EXTRACTS

5.1 HPTLC Fingerprint (FP) with marker compounds

Chromatography is used to create a chemical fingerprint of botanical products to identify and analyze their components (Tistaert *et al*., 2011). Fingerprinting can identify plants, measure chemically active compounds, and identify pesticides. TLC is used to visually describe and separate botanical medications to assess their quality (Salmon *et al*., 2012; Tistaert *et al*., 2011; Vander Heyden, 2008). High-performance thin-layer chromatography has advanced TLC technology. HPTLC provides qualitative and quantitative capacity data (Karpinski, 2010). HPTLC reduces solvent usage and may evaluate multiple samples at once, saving time and money (Dharmishtha *et al*., 2009; Verma & Gavankar, 2018). HPTLC usually produces more accurate and trustworthy results. Gradient mobile phases and detection methods are also key fingerprinting aspects because HPTLC technology is changing frequently. This evaluation is essential for determining HPTLC's quality control efficacy and exploring its potential uses. HPTLC's capacity to run many samples simultaneously and form colored bands on the plate is a major benefit. This facilitates standard identification and sample comparisons (Verma & Gavankar, 2018). Derivatization is often needed to observe samples under specified light wavelengths. Sulfuric acid is a universal agent for this, although it degrades chlorogenic acid, reducing its visibility. Pre- and post-derivatization plate pictures provide a comprehensive dataset for analysis. HPTLC is a good choice for improving chromatographic fingerprints since it offers better separation and resolution than TLC. HPTLC and digital scanning profiling provide on-site qualitative and quantitative scanning densitometry measurements. HPTLC can identify plants by their secondary metabolites and evaluate crude extracts, making it important for plant taxonomy (Khan *et al*., 2011). HPTLC fingerprinting is a reliable method for identifying herbal drugs because to its clear and precise results and precision (Cortés *et al*., 2014). It aids herbal product quality control by recognizing adulterants (Teo *et al*., 2013). Using HPTLC, a number of market-available pharmaceutical formulations have been evaluated (Gandhi *et*

al., 2012; Meena & Sandhya, 2013; Patel *et al*., 2013). Using marker molecules from abundant but not always biologically active substances to standardize herbal medicine can yield inaccurate results (Ruiz *et al*., 2016). Herbal remedies are frequently considered safer than synthetic treatments, yet their lack of limits has led to many hazardous instances, underscoring the necessity for stringent management (Ruiz *et al*., 2016). Herbal medicine contamination and adulteration are growing concerns (Posadzki *et al*., 2013). Some countries have laws to ensure herbal medication safety, yet barriers remain. The WHO said that over 90% of member states and nations have herbal medicine rules in 2018 (Organization, 2019). Fingerprinting analyzes the entire chemical profile of plant specimens, making it more comprehensive. These methods produce detailed profiles and patterns linked to biological or pharmacological activity when applied with multivariate data analysis or chemometrics. This provides a trustworthy standard for assessing materials and formulations (Kharbach *et al*., 2020). Fingerprinting helps identify original and adulterated herbal medicines and plant species (Sima *et al*., 2018; Wang & Yu, 2015). HPLC and TLC are used to make chemical fingerprints. For the same reasons, molecular spectroscopy, capillary electrophoresis, mass spectrometry, and DNA-based technologies can obtain chemical or molecular fingerprints. Khan *et al*. (2011) and Misra *et al*. (2014) found HPTLC to be a phytochemical marker. Cortés *et al*. (2014) say it's good at recognizing plants by secondary metabolites (Khan *et al*., 2011; Misra *et al*., 2014). The method is commonly considered rational and useful for herbal drug quality control (Ram *et al*., 2011) and adulterant detection (Attimarad *et al*., 2011). Govindarajan *et al*. (2007) developed a simple thin-layer chromatography (TLC) method for identifying two compounds in *Heracleum candicans* roots. HPTLC fingerprint profiles, which contain chemical patterns, can identify plant species like *Heracleum rigens*. These characteristics help identify and verify plant material, ensuring quality and consistency in traditional medicine and herbal products. Our study illustrated that both ethyl acetate extract and chloroform extract of the aerial parts of the plant contain xanthotoxin and psoralen marker compounds. This method can be highly beneficial in the identification as well as standardization of the plant based on our results.

5.1.1 Experimental

5.1.1.1 Description of samples used in analysis

The marker compounds i.e. xanthotoxin (M1), psoralen (M2) were used in single band applications along with various extracts of *H. candicans* aerial part using MP: Toluene: ethyl acetate (9:1), with humidity: 33% and temperature 25 ºC as shown in table 14.

Table 14: Description of samples used in analysis

Description	Volume
Pet ether extract	15.0 µl
Chloroform extract	15.0 µl
Ethyl acetate extract	15.0 µl
Methanol extract	10.0 µl
Hydroalcoholic extract	5.0 µl
M1 (Xanthotoxin)	1.0 µl
M2 (Psoralen)	2.0 µl

5.1.1.2 System setup for HPTLC

The system for HPLTC testing was setup included the use of software Server DESKTOP-O5JU7BQ, version 2.5.18262.1, Linomat 5 having S/N: 270115 and Visualizer 2 having S/N: 270089.

5.1.1.3 Chromatography set up conditions

A silica gel 60 F 254 plates, HPTLC grade (Merck) were used during the analysis. The setup of the chromatographic conditions involved the following parameters which are represented in table 15 as;

Table 15: Chromatography conditions

Parameter	Description
Stationary phase	Merck, HPTLC plates silica gel 60 F 254
Plate format	100.0 x 100.0 mm
Application type	Band
Application	Position Y: 8.0 mm, width:0.0 mm, length: 8.0 mm,
Track	First position X: 15.0 mm, distance: 11.4 mm
Solvent front position	70.0mm

5.1.1.4 Parameters settings for image clean plate

The following settings were applied for image clean plate 1a – Visualizer as represented in table 16.

Table 16: Parameters setting for image clean plate, developed plate and derivatized plate using 1a – Visualizer (S/N: 270089)

Quality	Standard
R White	Auto, level 85 %, Band
R 254	Auto, level 85 %, Band
R 366	Auto, level 85 %, Band
Instrument diagnostics	Valid diagnostics
Documentation step label	

5.1.1.5 Parameters setting for application 1- Linomat 5

Table 17: Parameters setting for application 1- Linomat 5 (S/N: 270115)

Parameter	Description
Sample solvent type	Methanol
Dosage speed	150 nl/s
Pre-dosage volume	0.20 µl
Instrument diagnostics	Valid diagnostics

5.1.1.6 Parameters setting for development 1 - chamber

Table 18: Parameters setting for development 1 - chamber

Parameter	Description
Tank	TTC 20x10
Mobile phase	Toluene: ethyl acetate (9:1 v/v)
Saturation time	20 min
Use saturation pad	True
Use smart ALERT	False
Volume front through	10 ml
Volume rear through	20 ml
Drying time	5 min
Drying temperature	Room temperature

5.1.1.7 Parameters setting for Derivatization 1 - dip

Table 19: Parameters setting for derivatization 1 – dip using Anisaldehyde-sulfuric acid

Parameter	Description
Dipping speed	5
Dipping time	0 sec
Reagent preparation	-
Heating	105ºC for 3 min

5.1.2 Results of evaluation-1 for various samples

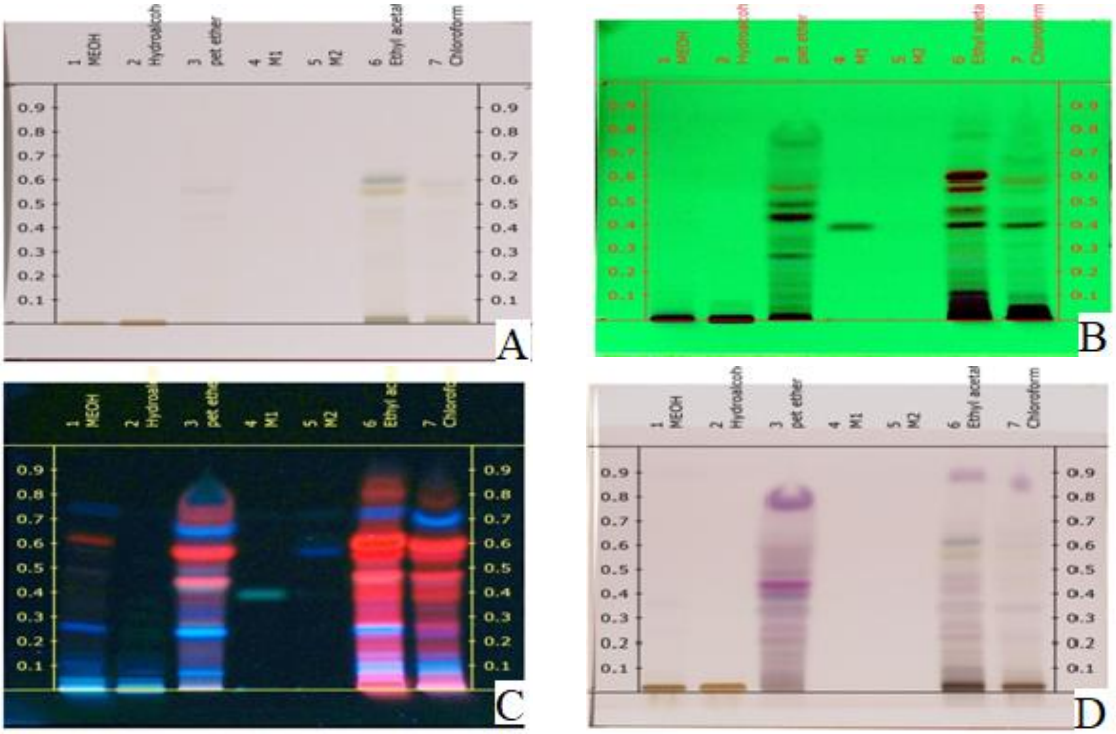


Figure 11: Developed remission of various samples **A-D: (A=** Remission at white visible wavelength of underivatized; B= Remission at 254 nm wavelength of underivatized; C= Remission of various samples at 366 nm wavelength of underivatized; D= Derivatized plate under White-Visible remission.

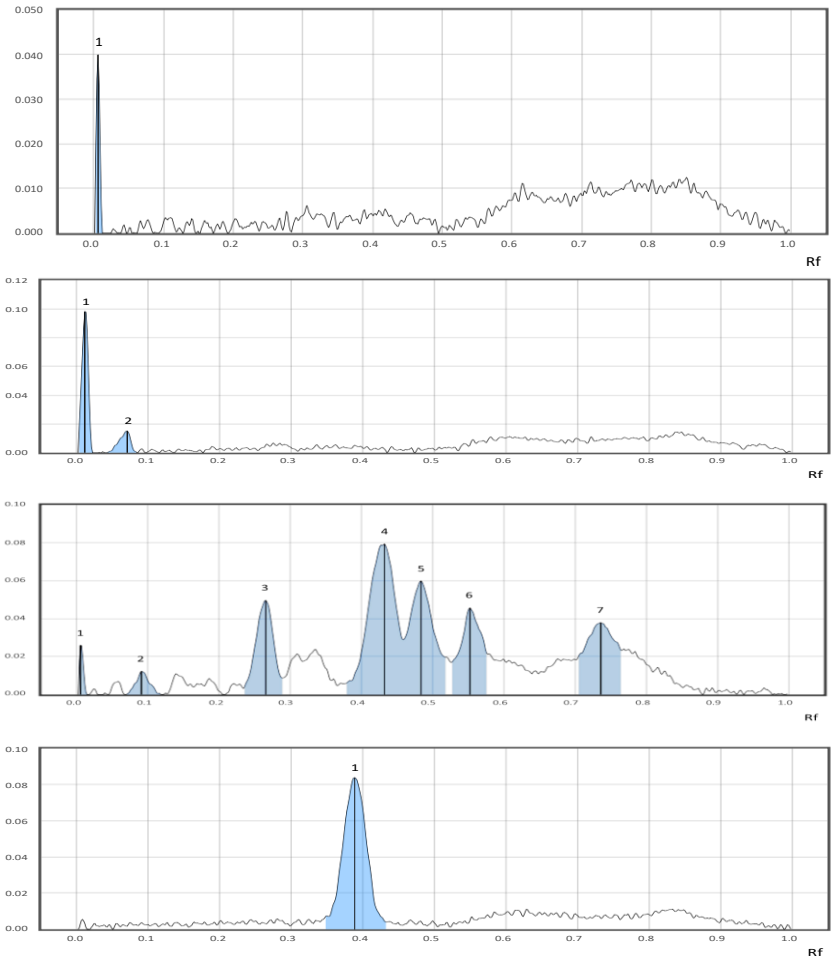
5.1.2.1 Integration parameters for evaluation

The integration parameters for the evaluation are indicated in the table 20.

Table 20: Integration parameters for evaluation

Bounds	[0.000,1.000]
Smoothing	Savitzky–Golay of order 3 and window 7
Baseline correction	Lowest slope with noise 0.05
Profile subtraction	None
Peaks detection	Gauss (legacy) with sensitivity 0.1, separation 1 and threshold 0.1
Scan	Wavelength 254 nm

5.1.2.2 Results of the evaluation at 254 nm wavelength



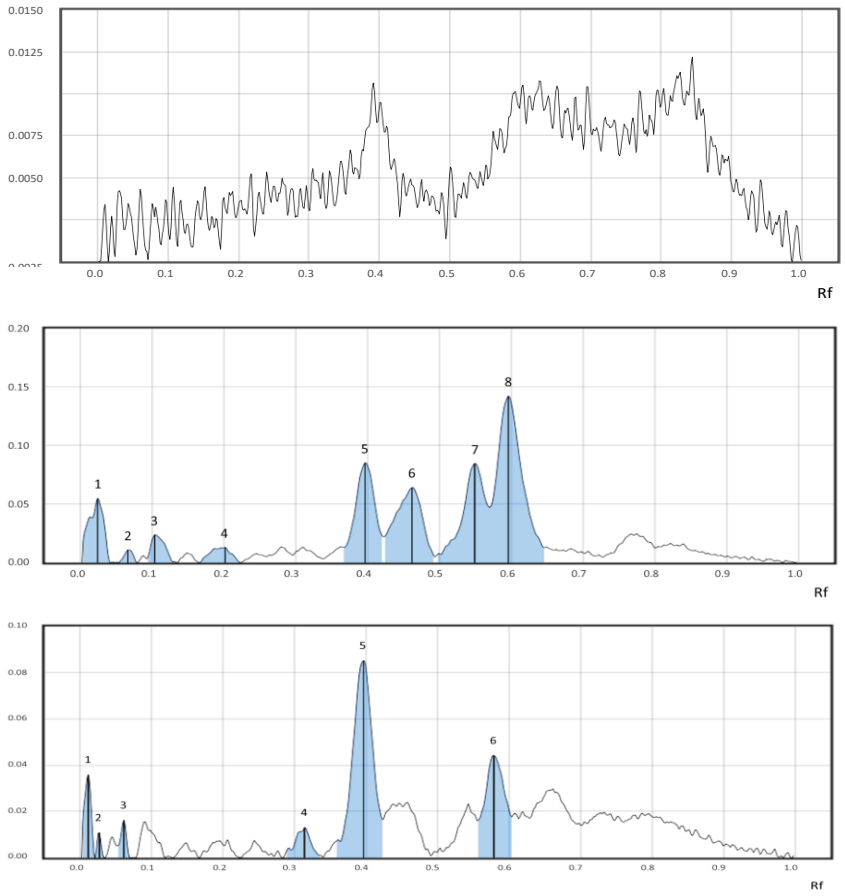
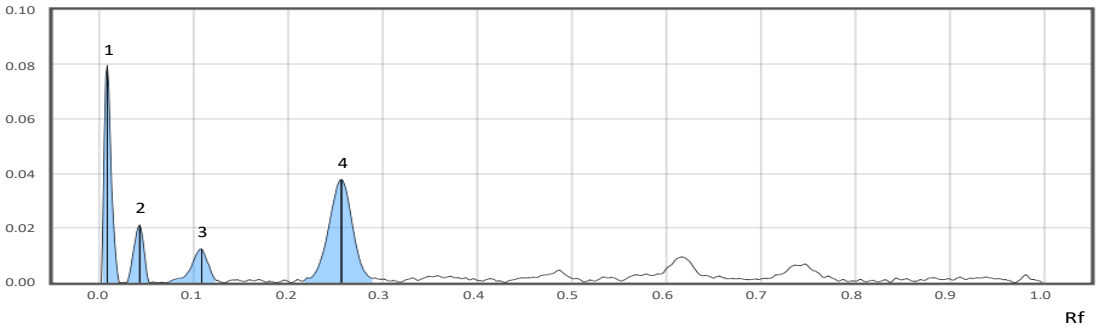


Figure 12: HPTLC Chromatograms of various extracts at 254 nm: (A= Methanol extract using 10.0 µl; B=Hydroalcoholic aerial extract using 5.0 µl; C= Pet. ether aerial extract using 1.0 µl; D= Marker compound xanthotoxin (M1) 1.0 µl; E= Marker compound Psoralen (M2) 2.0 µl; F= Ethyl acetate extract using 2.0 µl; G= Chloroform extract using 15.0 µl)

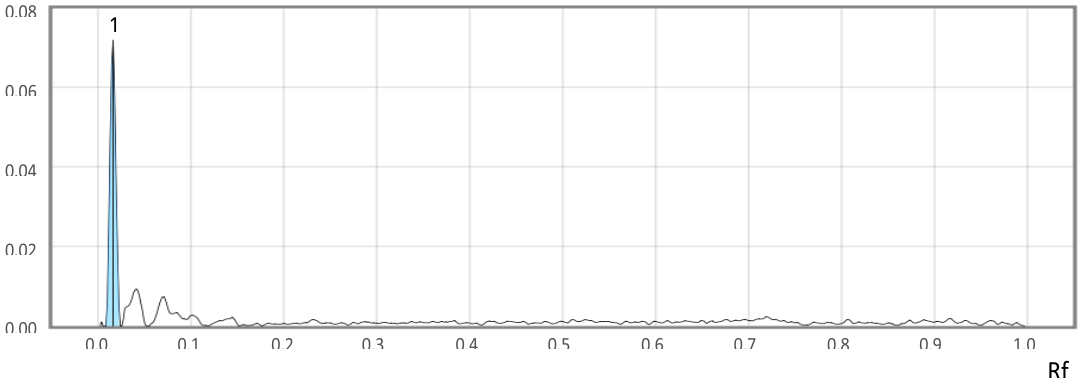
Table 21: Results of the evaluation at 254 nm wavelength

Extract	Peak	Start		Max			End		Area	
		R*f*	H	R*f*	H	%	R*f*	H	A	%
Methanol extract	1	0.001	0.0000	0.006	0.0399	100.0	0.012	0.0000	0.00024	100
Hydro-alcoholic extract	1	0.001	0.0000	0.011	0.0982	86.22	0.022	0.0000	0.00104	78.90
	2	0.040	0.0000	0.070	0.0152	13.38	0.083	0.0006	0.00028	21.10
Pet. Ether extract	1	0.001	0.0000	0.005	0.0259	8.34	0.014	0.0000	0.00017	1.50
	2	0.069	0.0000	0.090	0.0122	3.93	0.119	0.0000	0.00026	2.31
	3	0.234	0.0032	0.265	0.0497	15.97	0.289	0.0086	0.00146	12.91
	4	0.379	0.0061	0.432	0.0795	25.58	0.459	0.0289	0.00356	31.36
	5	0.459	0.0289	0.484	0.0599	19.28	0.518	0.0195	0.00240	21.13
	6	0.526	0.0169	0.552	0.0457	14.70	0.577	0.0212	0.00161	14.17
	7	0.704	0.0190	0.736	0.0379	12.19	0.766	0.0232	0.00189	16.62
Xanthotoxin (M1)	1	0.346	0.0062	0.388	0.0839	100.0	0.43	0.0039	0.00329	100.0
Psoralen (M2)	-	-	-	-	-	-	-	-	-	-
Ethyl acetate extract	1	0.001	0.0000	0.023	0.0548	11.46	0.040	0.0000	0.00133	7.84
	2	0.053	0.0000	0.065	0.0107	2.23	0.078	0.0000	0.00015	0.88
	3	0.092	0.0032	0.103	0.0238	4.96	0.128	0.0004	0.00052	3.10
	4	0.164	0.0000	0.201	0.0129	2.70	0.221	0.0000	0.00044	2.61
	5	0.367	0.0129	0.396	0.0852	17.81	0.421	0.0221	0.00273	16.16
	6	0.423	0.0220	0.462	0.0642	13.41	0.493	0.0050	0.00282	16.68
Chloroform extract	1	0.001	0.0000	0.011	0.0358	17.47	0.020	0.0000	0.00040	7.66
	2	0.020	0.0000	0.026	0.0107	5.24	0.034	0.0000	0.00007	1.32
	3	0.051	0.0051	0.061	0.0162	7.89	0.069	0.0000	0.00015	2.95
	4	0.289	0.0029	0.314	0.0130	6.32	0.337	0.0008	0.00032	6.17
	5	0.357	0.0069	0.396	0.0852	41.54	0.423	0.0166	0.00280	53.64
	6	0.555	0.0160	0.579	0.0442	21.53	0.604	0.0176	0.00148	28.26

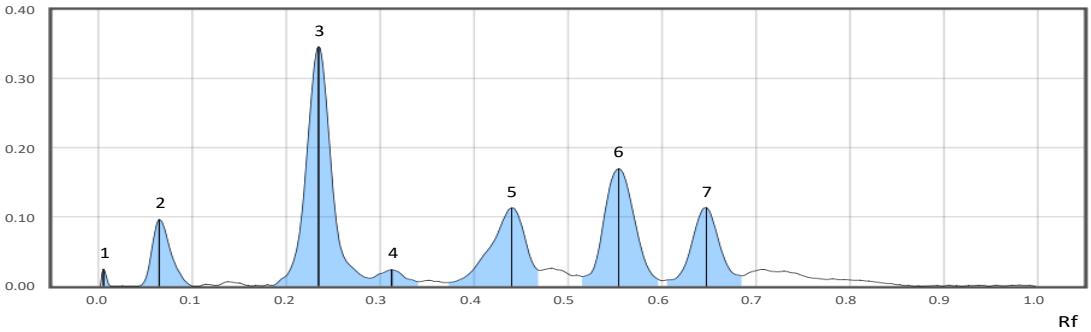
5.1.2.3 Results of evaluation-2 using wavelength at 366 nm wavelength



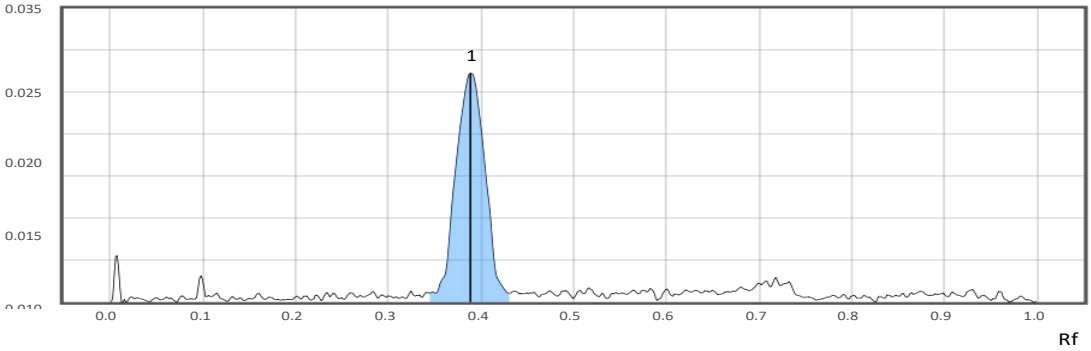
A



B



C



D

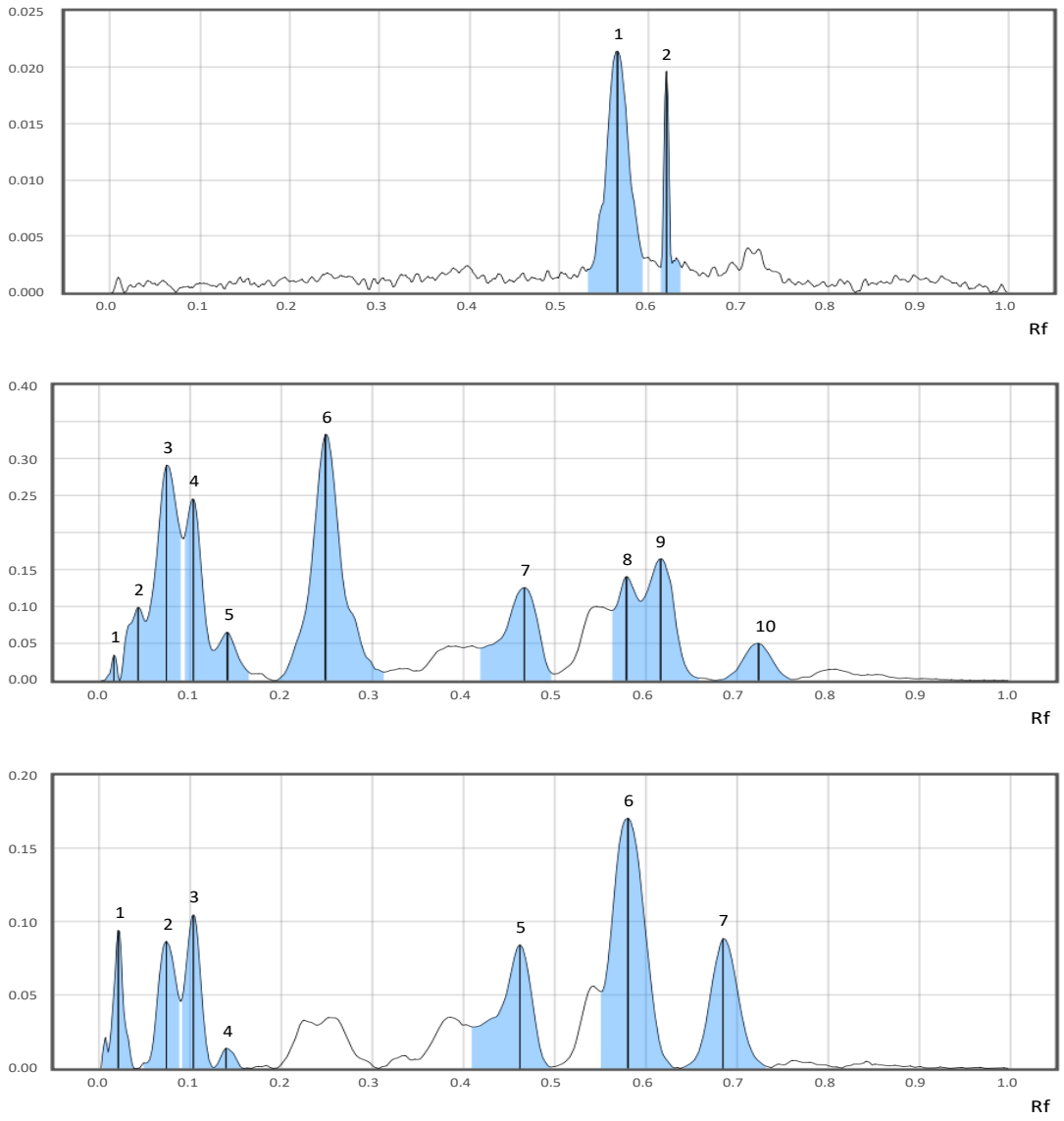


Figure 13: Results of HPTLC Chromatograms for various extracts at 366 nm: (A= Methanol extract using 10 µl; B= Pet. ether extract using 15 µl; C= Hydroalcoholic extract using 15 µl; D= Xanthotoxin using 1.0 µl; E= Psoralen using 2.0 µl; F= Ethyl acetate extract using 15 µl; G= Chloroform extract using 15 µl.

Table 22: Results of the HPTLC evaluation of various extracts and marker compounds @366nm

Extract	Peak	Start		Max			End		Area	
		R*f*	H	R*f*	H	%	R*f*	H	A	%
Methanol extract	1	0.001	0.0000	0.008	0.0796	52.75	0.020	0.0000	0.00069	29.74
	2	0.028	0.0000	0.042	0.0211	13.98	0.053	0.0000	0.00026	11.05
	3	0.070	0.0000	0.108	0.0124	8.21	0.123	0.0007	0.00026	11.02
	4	0.215	0.0005	0.256	0.0378	25.07	0.289	0.0015	0.00112	48.20
Hydro-alcoholic extract	1	0.008	0.0000	0.016	0.0719	100.0	0.023	0.0000	0.00053	100.0
Pet ether extract	1	0.001	0.0000	0.005	0.0247	2.78	0.012	0.0000	0.00013	0.45
	2	0.042	0.0000	0.064	0.0966	10.87	0.101	0.0000	0.00221	7.46
	3	0.184	0.0009	0.234	0.3468	39.02	0.287	0.0116	0.01076	36.40
	4	0.289	0.0114	0.312	0.0237	2.66	0.340	0.0063	0.00082	2.77
	5	0.368	0.0050	0.440	0.1135	12.77	0.470	0.0226	0.00503	17.02
	6	0.515	0.0137	0.554	0.1698	19.10	0.559	0.0077	0.00655	22.16
	7	0.602	0.0081	0.647	0.1138	12.80	0.685	0.0148	0.00406	13.74
Xanthotoxin	1	0.345	0.0010	0.388	0.0273	100.0	0.43	0.0010	0.00101	100.0
Psoralen	1	0.530	0.0019	0.565	0.0214	52.19	0.59	0.0030	0.00068	80.76
	2	0.613	0.0022	0.619	0.0196	47.81	0.63	0.0022	0.00016	19.24
Ethyl acetate extract	1	0.005	0.0000	0.016	0.0342	2.21	0.02	0.0000	0.00025	0.52
	2	0.022	0.0000	0.042	0.0991	6.39	0.05	0.0798	0.00197	4.10
	3	0.050	0.0798	0.073	0.2918	18.82	0.09	0.1930	0.00825	17.21
	4	0.092	0.1919	0.103	0.2456	15.84	0.12	0.0405	0.00525	10.95
	5	0.125	0.0405	0.140	0.0652	4.20	0.16	0.0089	0.00169	3.51
	6	0.193	0.0000	0.248	0.3334	21.50	0.31	0.0111	0.01314	27.40
	7	0.417	0.0439	0.466	0.1258	8.11	0.49	0.0084	0.00581	12.12
	8	0.562	0.0948	0.579	0.1407	9.07	0.59	0.1071	0.00388	8.09
	9	0.594	0.1071	0.616	0.1648	10.63	0.66	0.0031	0.00587	12.23
	10	0.677	0.0000	0.724	0.0501	3.23	0.76	0.0014	0.00185	3.86
Chloroform extract	1	0.001	0.0000	0.020	0.0942	14.64	0.037	0.0000	0.00128	6.59
	2	0.045	0.0014	0.073	0.0867	13.48	0.087	0.0477	0.00195	10.04
	3	0.089	0.0457	0.103	0.1048	16.30	0.125	0.0005	0.00204	10.51
	4	0.125	0.0005	0.139	0.0136	2.11	0.158	0.0000	0.00023	1.21
	5	0.409	0.0282	0.462	0.0843	13.11	0.496	0.0008	0.00362	18.63
	6	0.551	0.0523	0.580	0.1708	26.56	0.632	0.0003	0.00710	36.59
	7	0.638	0.0000	0.685	0.0886	13.78	0.736	0.0007	0.00319	16.43

5.1.3 Conclusion

This study presents a comprehensive HPTLC fingerprint analysis and phytochemical profiling of *Heracleum candicans* extracts from Pahalgam, Kashmir, India. The diverse chemical composition and identification of bioactive compounds highlight the potential of this less explored botanical resource for pharmaceutical and nutraceutical applications. The findings contribute to the growing body of knowledge on the rich plant diversity in the northern Himalayan region and emphasize the need for further research to unlock the therapeutic potential of *Heracleum candicans*. On the basis of HPTLC analysis, it can be confirmed that the ethyl acetate extract and chloroform extract of the plant contains marker compounds i.e. xanthotoxin and psoralen. This is the first such report that has been under taken on this plant species from the Himalayan range of Jammu & Kashmir, India. The data of the both the evaluations i.e. at a wavelength of 254 nm and 366 nm imply that chloroform and ethyl acetate extracts give consistent results on the presence of marker compounds xanthotoxin and psoralen which is in conformity with the earlier reports on the hptlc analysis of Heracleum plant species.

5.2 QbD-guided HPLC method development and validation

The medicinal phytonutrients found in plants and botanical extracts are extensive and diverse. Plant phenols have gained a lot of interest for their possible medicinal uses, thus people commonly take them (Kostova, 2005). Umbelliferone, psoralen, and eugenol are compounds found in a number of plant species; these compounds have diverse impacts on creatures from the plant and animal kingdoms (Lacy & O'Kennedy, 2004). Modern research has shown that coumarins are effective against a broad variety of pathologies, including bleeding, acne, dermatitis, bacterial infections, mollusk killing, pain relief, vasodilation, and hypothermia. According to a 1964 study by Soine, coumarins exhibit anti-oxidative and antiproliferative qualities that are truly extraordinary (Soine, 1964).

Bergapten, isopimpinellin, Imperatorin bya-kangelicol, phellopterin, and xanthotoxin, are among the furanocoumarins that the Heracleum genera are famous for synthesizing and these chemicals can affect many different parts of the body (Trott *et al*., 2008). Drugs

such as these are crucial in treating vitiligo and psoriasis. Furocoumarins are prone to photo toxicity and photo dermatitis when exposed to UV radiation (Ashwood-Smith *et al*., 1980). The ability of furocoumarins produced by *H. candicans* to increase the skin's sensitivity to light has led to their extensive usage in medical treatments (Devagiri *et al*., 1996). In 2002a, Mukherjee investigated the unsightly properties of furocoumarin in *H. candicans* rhizomes (Pulok K Mukherjee, 2002). Ashwood-Smith *et al*. (1980) noted that these compounds can function as phototoxic chemicals, leading to photo dermatitis when exposed to UV radiation (Ashwood-Smith *et al*., 1980). The formation of reactive oxygen species can be damaging to lysosomes when furocoumarins bind to biological components such as lipids and amino acids. Another possible outcome of this interaction is the covalent modification of peptides, which could result in the creation of novel antigens (Schmitt *et al*., 1995). Because of its capacity to increase the skin's sensitivity to light, furocoumarins derived from *H. candicans* find extensive application in the pharmaceutical business (Devagiri *et al*., 1996). Similarly, a technique for the quantitative determination of Furocoumarins in fruits of *H. candicans* using HPLC was investigated (Govindarajan *et al*., 2007). The significance of furocoumarins makes quantitative measurement of their levels in *H. candicans* essential. The purpose of the TLC analysis carried out was to determine the concentrations of furocoumarins in *H. candicans* shoots (Devagiri *et al*., 1996). Several researches have tried to detect furocoumarins using HPLC in different forms, such as capsules and tablets (Andréa *et al*., 2006). The presence of furocoumarins has also been detected in citrus essential oils and other plant sources (Dugo *et al*., 2000; Frérot & Decorzant, 2004). We achieved a first by standardizing *H. candicans* with xanthotoxin as the pharmacological reference.

5.2.1 Experimental

Xanthotoxin and psoralen of HPLC grade were purchased from Hychem Laboratories through *Carbanion.com*. Acetonitrile (ACN), glacial acetic acid, and methanol, all of which are of the highest purity analytical grade, were acquired from M/s Fisher Scientific in Mumbai, India, and used throughout the research. For all analytical purposes, doubly distilled water was adopted.

5.2.1.1 Analysis tools and environments

5.2.1.1.1 Chromatographic equipment and working environment

We used a Waters e2695 Separation Module RP-HPLC system (Milford, MA, USA) equipped with a 2998 PDA detector to develop the analytical approach. A Hibar PurospherVR® STAR column developed by Merck in Darmstadt, Germany, with dimensions of 5 mm × 250 × 4.6 mm, was used in the HPLC experiments. A combination of acetonitrile (ACN) and water made up the mobile phase, which was pH-lowered to 3 with the use of glacial acetic acid. The outcomes were assessed employing a database application for chromatography.

5.2.1.1.2 Standard solution preparation

A stock solution with a functional strength of 100 $\mu g \cdot ml^{-1}$ was prepared by adding a carefully determined quantity of xanthotoxin (10 mg) to water in the mobile phase. By neutralizing and filtering the material through a thin membrane, different quantities of xanthotoxin were obtained, spanning from 0.1 to 100 $\mu g \cdot ml^{-1}$.

5.2.1.2 HPLC method development QbD-enabled

5.2.1.2.1 Setting analytical target profile (ATP) and CAAs

As a prerequisite to developing a methodology for analysis according to Quality by Design (QbD) guidelines, the needed criterion was determined to be the Analytical Target Profile (ATP) with its particular characteristics (Sandhu *et al*., 2016). The peak area (PA), retention time (RT), tailing factor (TF), and theoretical plate count (PC) were determined to be the CAAs, which stand for ATP, following a comprehensive literature review and group brainstorming sessions (A. Jain *et al*., 2019).

5.2.1.2.2 Risk assessment studies

To keep the HPLC technique efficient, it is necessary to optimize the chromatographic solution after doing an initial risk assessment of any potential hazards elements associated with this procedure. Minitab® 17 software (Minitab, LLC, USA), was used to build an Ishikawa fishbone diagram, a cause and effect diagram, to show the linkages that exist among ATP's CMPs and CAAs. The characteristics were precisely defined as low-, moderate-, or high-risk using a rigorously constructed REM (Beg *et al*., 2016; B. Singh *et al*., 2005).

5.2.1.2.3 Factor optimization studies

Two variables—mobile phase Design Expert® 11—were shown to have an economically significant impact after CMPs conducted risk evaluation and component screenings. Table 24 displays the predictive matrix used for 17 experimental runs, five of which were repeated at the center point (0, 0). The chosen CMPs had been set to high (+1), medium (0), and low (-1) levels. X1 and X2 were tested by the use of an FA solution (20 μg•mL−1) to determine their effects on the selected CAAs, specifically PA, RT, TF, and PC.

5.2.1.2.4 Optimization data analysis and model validation

Dataset was analyzed utilizing Design Expert® 11's Multiple Linear Regression Analysis (MLRA) tool. The variables influencing and output were established using a second-order polynomial (quadratic) model. Analysis of variance (ANOVA), coefficient of correlation (r), and lack of fit were all applied to the hypothesis (Beg *et al*., 2016). Through a progressive analysis of the likely components utilizing the outcomes of the experiment, possible causal attribution agents (CAAs) and causal mechanism processes (CMPs) were discovered. The method operable design region (MODR), often called design space, was created using the goals of each CAA. To achieve this, we used the Fractional Factorial Central Composite Design (FCCD) to analyze the collected data and conform it to the runs of testing. Validation of methods followed MODR verification. By comparing the 3D-response surface plot with the equivalent 2D-contour plots, the factor-

response relationships may be visually examined and understood (Beg *et al*., 2016; de Souza Anselmo *et al*., 2019).

5.2.1.2.5 Determination of optimum chromatographic solution

A statistical optimization method was adapted to determine the optimal chromatographic resolution for different CAAs. The objective here was to maximize PA and PC values simultaneously minimizing TF and RT values. The most favorable chromatographic solution in the provided design space has been established by a graphical technique (Dalvi *et al*., 2018; B. Singh *et al*., 2005). Optimization was performed as well thereafter.

5.2.1.3 Analytical method validation

For regulatory compliance and ongoing usefulness spanning scientific purposes, standardization of the xanthotoxin analytical method is of the utmost importance. This technique was thoroughly evaluated according to ICH Q2 (R1) guidelines and showed that it was linear with respect to the measured amount of Xanthotoxin (I. C. H. H. T. Guideline, 2005). Consistent and repeatable quantification was guaranteed by evaluating precision and accuracy. The ability of the approach to distinguish xanthotoxin from possible interferences was confirmed by specificity confirmation. Evaluations of its robustness confirmed that it remains consistent in different environments. Its sensitivity was determined by determining the Limit of Detection (LOD) and Max Quantification (LOQ). The analytical technique is supported in its usefulness for varied uses including research, quality control, and clinical investigations, by this extensive validation that underlines its reliability, predictability, and repetition.

5.2.1.3.1 Quality control samples preparation

The following different concentrations of quality assurance samples were painstakingly prepared: 4.8 µg/ml for the low quality 6 µg/ml for the intermediate, and 7.2 µg/ml for the highly effective control. After being prepared, these specimens were carefully maintained at 4°C until they were ready for evaluation. The meticulously produced samples were filtered using a 0.22 µM syringe filter to guarantee the highest level of purity and accuracy in the analysis procedure prior moving onto a chromatography.

5.2.1.3.2 Linearity and range

In order to assess the uniformity of the approach to analysis, a total of five samples were prepared, with quantities varying from a 2 to 10 µg/ml using a stock solution of 1000 mg/ml. The Y-axis of the linearity curve represents peak area (PA) in millimeters per square meter, while the X-axis represents xanthotoxin level in mg/ml. To quantify the method's performance, we used the MS-Excel 365 software for enterprise to calculate slope, regression equation, and regression coefficient (r^2), which are important characteristics for describing the curve's linearity.

5.2.1.3.3 LOD and LOQ

The method for determining the Limit of Detection (LOD) and Limit of Quantification (LOQ) involved assessing signal-to-noise ratios at 3:1 and 10:1 using SD and the slope of the curve. The calculation of LOD and LOQ was carried out using the formula:

$$LOD = 3.3 \times \frac{\sigma}{S} \text{ and } LOQ = 10 \times \frac{\sigma}{S}$$

Where, σ represents the standard deviation, and S denotes the slope of the standard curve.

5.2.1.3.4 Accuracy

The absolute recoveries at MQC, LQC, and HQC levels —representing recovery at 80%, 100%, and 120%, respectively—were studied as part of the preciseness of the measurement procedure for xanthotoxin. Every collection had its data mean, standard deviation, percentage of relative standard deviation (% RSD), and percentage of absolute retrieval computed after the research procedure was repeated five times. The data had to be accurate within certain bounds, and our study was trying to do just that. An efficient but systematic methodology was used to evaluate the accuracy of the xanthotoxin analytical method by determining the % absolute recovery using a formula provided in Equation.

$$\% \text{ Absolute recovery} = \frac{Actual\ concentration\ recovered}{Theoritical\ concentration} \times 100$$

5.2.1.3.5 Precision

We conducted thorough evaluations of the inter-day reproducibility, intraday repeatability, and inter-analyst consistency of xanthotoxin analytical method that was established. To assess interday precision, five injections were administered on the first day at three dissimilar quantities (MQC, LQC, and HQC) and then repeated for three days in a row. To further assess intraday precision, quintuplicate samples were injected on the same day. Furthermore, the inter-analyst precision was examined by having three analysts inject five samples of MQC on the same day. The analytical procedure was applied to the recoveries, standard deviations, percentages of RSD, and derived mean areas.

5.2.1.3.6 Robustness

Analytical robustness is an assessment of how well a procedure holds up when exposed to deliberate changes to critical variables. A range of flow rates (1.0, 0.8, and 0.6 ml/min) and wavelengths (250, 254, and 258 nm) were employed to assess the method's robustness for testing xanthotoxin. A xanthotoxin MQC solution was used in the course of the experiment. From the chromatogram information we were in a position to estimate the mean area, mean retention time, and % RSD (González & Herrador, 2007).

5.2.1.3.7 System Suitability

To make sure that the chromatographic setup is appropriate for xanthotoxin analysis, System Suitability Testing (SST) checks the system's resolution, column efficiency, and repeatability. The test included injecting five 10 µg/ml working solution injections and was constructed on a comprehensive review of samples, electronics, analytical techniques, and equipment. The following characteristics were derived: percent relative standard deviation, theoretical plates, retention time, peak area, and height equivalent to theoretical plate (HETP). In order to determine the analytical method's robustness and reliability for xanthotoxin within specified variables, the outcomes obtained were compared against its standard ranges.

5.2.2 Results

5.2.2.1 RP-HPLC Method Development Preliminary Studies

It has been established using an AQbD paradigm to quantitatively estimate xanthotoxin in both bulk and nano formulation. The initial studies followed the standard operating procedures outlined in the relevant literature and pharmacopoeias. A number of attempts were attempted to reduce peak tailing, improve method effectiveness, and increase peak resolution. Researchers looked into changes in pH (1.5, 1.7, and 1.9), flow rate (0.8, 1.0, and 1.2 ml/min), and the make-up of the mobile phase (methanol, acetonitrile, and 0.1% v/v OPA). Tailing factor, peak resolution, retention duration, and theoretical plates, all remained unsatisfactory in spite of numerous experiments. A peak could not be observed when acetonitrile along with 0.1% v/v OPA was mixed. Finally, the best mobile phase was found to be a mixture of ACN and water; it produced a peak with good retention time, resolution, theoretical plates, and peak tailing. To further develop the xanthotoxin robust RP-HPLC method, chromatographic settings were refined using aBBD, guaranteeing the method's dependability and precision.

5.2.2.2 Risk Analysis Studies

To follow the rules of value risk supervision, we must do risk assessment evaluations to find out the different causes and sub-causes of variation and how they might be connected with important analytical qualities. Table 23 shows the results of the risk assessment for many important criteria. These evaluated parameters provide an explanation for the interaction between various analytical qualities and procedure parameters. The process of creating a risk estimation matrix began with a thorough probability and criticality analysis of quite a few input factors. This evaluation was based on pertinent publications and the different levels of risk linked to each technique parameter. The mobile phase ratio, pH, and flow rate were determined to be high-risk factors, notwithstanding the examination of all other parameters. Alternatively, other parameters, including column temperature, injection volume, and type of flow were

deemed low-risk, indicating that they had minimal impact on the method's effectiveness, in contrast to the medium-risk provided by the column dimension. From the results from risk and screening evaluations, a methodical strategy for continued enhancement was put in place. Modeling, experimental designs, and optimal search with reaction surface approaches were used to discover the most advantageous approach to construct the computational design realm. Using experimental design to improve things also helped lower risks by making it easier to guess how vulnerable something would be and how strong it would be in relation to the xanthotoxin input parameters.

Table 23: Outlines the parameters used in assessing the risk associated with the development of a resilient analytical method

CAAs	Mobile phase: Composition	pH: 0.1% OPA	Injection: Volume	Flow: Rate
Peak area	-	+	-	+
Retention time	-	+	-	+
Peak height	+	-	-	+
Tailing factor	-	-	-	-

5.2.2.3 Optimization

Xanthotoxin in bulk was separated using chromatographic settings that were made optimal using a Design of Experiments (BBD) technique. The mobile phase ratio (ACN: water), pH, and flow rate at low (-1), medium (0), and high (+1) levels were the three critical variables that were taken into account. In order to find the perfect circumstances, seventeen trial runs had been carried out, five of which were central points. With each test, we tracked retention time (R1), peak area (R2), peak height (R3), tailing factor (R4), and theoretical plates (R5). The research led to the determination that a mobile phase ratio of 1.8% v/v, a pH of 6, and a flow rate of 0.8 ml/min were the ideal chromatographic parameters. With a desirability rating of 0.773, these parameters are quite close to the ideal chromatographic setup.

Table 24: A Box-Behnken design displaying how uncorrelated factors impact the dependent factor at low, moderate, and higher levels

	Factor			Responses				
Run	A: Ratio of Mobile phase	B: pH	C: Flow rate (ml/min.)	(R1): Retention time (min.)	(R2): Peak area (mAu)	(R3): Peak height (AU)	(R4): Tailing Factor	(R5): Theoretical Plate (cm)
1	-1	0	-1	9.3	242594	12775	1.077	5264.08
2	-1	-1	0	5.3	200456	15455	1.029	5232.25
3	0	+1	-1	8.3	220474	14030	1.232	5956.08
4	+1	+1	0	7.7	203904	15515	1.017	7332.35
5	+1	0	+1	4.5	156881	14543	1.043	5669.94
6	0	0	0	6.5	208186	15604	1.131	6466.94
7	0	0	0	5.6	165376	14553	1.105	5083.51
8	0	0	0	5.6	182327	13683	1.123	5179.63
9	0	-1	-1	8.4	200097	12886	1.232	6125.59
10	0	0	0	5.6	199273	13838	1.115	5477.48
11	-1	0	+1	5.8	209612	15844	1.003	4285.19
12	0	+1	+1	7.6	194638	13381	1.025	5896.08
13	-1	+1	0	7.5	231470	11640	1.102	3150.81
14	0	-1	+1	7.2	200216	14348	1.225	5751.49
15	0	0	0	5.6	149726	13990	1.119	5619.05
16	+1	0	-1	4.8	163360	13625	1.014	7686.85
17	+1	-1	0	5.2	107174	12306	1.031	6941.23

Table 25: Factor optimization at various levels

Factors	Levels		
	Low (-1)	Middle (0)	High (+1)
Mobile phase ratio (A : B)	1.6	1.8	2.0
pH	5	6	7
Flow rate (ml/min.)	0.6	0.8	1.0

All measured values for critical variables in the xanthotoxin study remained within the intended range. The best chromatographic conditions were used in the following HPLC analysis after graphical optimization was implemented to find the ideal design space. Critical responses that include retention time, peak area, tailing factor, and theoretical plates were examined in the xanthotoxin chromatogram utilizing the optimal approach. The results from different investigations were analyzed using a variety of kinetic models, such as linear, second order (2FI), quadratic, and cubic models. Retention time and peak area were best fit by the quadratic model ($p < 0.0001$), whereas peak height, tailing factor, and theoretical plate were best fit by linear models ($p < 0.05$). A strong correlation of 0.9970 was found between the data collected and the kinetic results for independent variables that included both linear and quadratic models. According to Table 26, these results confirm that independent variables have both combined and individual effects on dependent variables. As shown in figure 14, contour plots and 3D plots were used to further clarify the effects of factors on responses.

Table 26: Presents the outcomes of the analysis of variance (ANOVA) for multiple regression models

Summary										
	Sequential p value					Lack of fit p value				
Source	R1	R2	R3	R4	R5	R1	R2	R3	R5	R5
Linear	0.1270	0.0043	0.6084	0.6015	0.0005	0.0106	0.6217	0.1380	0.0002	0.3917
2FI	0.7087	0.4704	0.0035	0.5680	0.1963	0.0069	0.5934	0.6117	0.0002	0.4783
Quadratic	0.0924	0.5501	0.5035	0.0061	0.8593	0.0124	0.4972	0.5461	0.0016	0.2720
Cubic	0.0124	0.4972	0.5461	0.0016	0.2720	-	-	-	-	-
Sequencing Approach Sum of Squares (Type 1)										
Source			Sum of squares					DF		
Mean Vs total	718.25	6.159×10^{11}	3.332×10^{09}	20.40	5.548×10^{08}	1	1	1	1	1
Linear Vs mean	11.25	1.106×10^{10}	2.912×10^{06}	0.0123	1.359×10^{07}	3	3	3	3	3
2FI Vs Linear	2.64	1.424×10^{09}	1.460×10^{07}	0.0145	1.823×10^{06}	3	3	3	3	3
Quadratic Vs 2FI	10.82	1.284×10^{09}	1.464×10^{06}	0.0555	3.117×10^{06}	3	3	3	3	3
Cubic Vs Quadratic	7.22	1.634×10^{09}	1.509×10^{06}	0.0124	1.710×10^{06}	3	3	3	3	3
Residual	0.6480	2.297×10^{09}	2.449×10^{06}	0.0004	1.204×10^{06}	4	4	4	4	4
Total	750.83	6.336×10^{11}	3.355×10^{09}	20.50	5.735×10^{08}	17	17	17	17	17
Lack of fit tests										
Linear	20.68	4.341×10^{09}	1.758×10^{07}	0.0825	3.845×10^{06}	9	9	9	9	9
2FI	18.04	2.918×10^{09}	2.973×10^{06}	0.0679	2.022×10^{06}	6	6	6	6	6
Quadratic	7.22	1.634×10^{09}	1.509×10^{06}	0.0124	1.710×10^{069}	3	3	3	3	3
Cubic	0.0000	0.0000	0.0000	0.0000	0.0000	0	0	0	0	0
Pure error	0.6480	2.297×10^{09}	2.449×10^{06}	0.0004	1.204×10^{06}	4	4	4	4	4
Model summary statistics										
Source			Standard deviation					R^2		
Linear	1.28	22597.15	1241.19	0.0798	623.20	0.3452	0.6249	0.127	0.1290	0.7292
2FI	1.37	22835.37	736.35	0.0826	568.00	0.4264	0.7054	0.7636	0.2819	0.8269
Quadratic	1.06	23696.12	751.96	0.0427	645.26	0.7584	0.7779	0.8275	0.8657	0.8437
Cubic	0.4025	23963.05	782.47	0.0096	548.73	0.9801	0.8702	0.8932	0.9961	0.9354
Using DOE models- Fit summary and ANOVA										
Summary										
Source			Adjusted R^2					Predicted R^2		
Linear	0.1941	0.5384	-0.0745	-0.072	0.6667	-0.274	0.3639	-0.701	-0.792	0.5010
2FI	0.0823	0.5286	0.6218	-0.148	0.7231	-1.548	0.1058	0.3103	-2.560	0.3933
Quadratic	0.4478	0.4924	0.6056	0.6930	0.6427	-2.578	-0.679	-0.219	-1.092	-0.568
Cubic	0.9204	0.4809	0.5730	0.9844	0.7416	-	-	-	-	-

Table 27: Summary of p-values and F-values

Source					Mean square			F-value				p-value		
Mean Vs total	718.25	6.159×10^{11}	3.332×10^{09}	20.40	5.548×10^{08}	-				-				
Linear Vs mean	3.75	3.687×10^{09}	9.708×10^{05}	0.0041	4.531×10^{06}	2.28	7.22	0.6302	0.6420	11.67	0.1270	0.0043	0.6084	0.6015	0.0005
2FI Vs Linear	0.8817	4.746×10^{08}	4.868×10^{06}	0.0048	6.076×10^{05}	0.4718	0.9101	8.98	0.7097	1.88	0.7087	0.4704	0.0035	0.5680	0.1963
Quadratic Vs 2FI	3.61	14.280×10^{08}	4.880×10^{05}	0.0185	1.039×10^{05}	3.21	0.7622	0.8631	10.14	0.2495	0.0924	0.5501	0.5035	0.0061	0.8593
Cubic Vs Quadratic	2.41	5.445×10^{08}	5.030×10^{05}	0.0041	5.700×10^{05}	14.86	0.9483	0.8216	44.56	1.89	0.0124	0.4972	0.5461	0.0016	0.2720
Residual	0.1620	5.742×10^{08}	6.123×10^{05}	0.0001	3.011×10^{05}	-				-					
Total	44.17	3.727×10^{10}	1.974×10^{08}	1.21	3.373×10^{07}	-				-					
Lack of fit test															
linear	2.30	4.824×10^{08}	1.953×10^{06}	0.0092	4.272×10^{05}	14.19	0.8400	3.19	98.76	1.42	0.0106	0.6217	0.1380	0.0002	0.3917
2FI	3.01	4.863×10^{08}	4.955×10^{05}	0.0113	3.370×10^{05}	18.56	0.8468	0.8093	122.01	1.12	0.0069	0.5934	0.6117	0.0002	0.4783
Quadratic	2.41	5.445×10^{08}	5.030×10^{05}	0.0041	5.700×10^{05}	14.86	0.9483	0.8216	44.56	1.89	0.0124	0.4972	0.5461	0.0016	0.2720
Cubic															
Pure Error	0.6480	5.742×10^{08}	6.123×10^{05}	0.0001	3.011×10^{05}										
Model summary statistics															
Source					Adjusted R^2			Predicted R^2			PRESS				
Linear	0.1941	0.5384	-0.0745	-0.0720	0.6667	-0.2747	0.3639	-0.7013	-0.7923	0.5010	41.53	1.126×10^{10}	3.903×10^{07}	0.1705	9.302×10^{06}
2FI	0.0823	0.5286	0.6218	-0.1489	0.7231	-1.5483	0.1058	0.3103	-2.5609	0.3933	83.02	1.583×10^{10}	1.582×10^{07}	0.3387	1.131×10^{07}
Quadratic	0.4478	0.4924	0.6056	0.6930	0.6427	-2.5780	-0.6797	-0.2194	-1.0926	-0.5686	116.57	2.973×10^{10}	2.797×10^{07}	0.1991	2.924×10^{07}
Cubic	0.9204	0.4809	0.5730	0.9844	0.7416	-	-	-	-	-					

5.2.2.4 Impact of independent factors towards dependent ones

5.2.2.4.1 Influence of independent variables on retention time

With a 12.61% chance that such a big F-value might result only from variations at random, the model may lack significance relative to intrinsic noise, as suggested by the Model F-value of 2.44. None of the model terms are statistically significant, even though generally, P-values < 0.0500 suggest the relevance of model terms. If the values of the model terms are more than 0.1000, it is possible that they are not significant. Model reduction could be explored to improve overall efficacy if a large number of terms are determined to be inconsequential, except those required for hierarchy support. In addition, the noteworthy significance of the 14.86 Lack of Fit F-value is highlighted by the fact that there is only a 1.24% chance that such a large Lack of Fit F-value might be due to noise. As seen in Table 28, a notable absence of fit is undesirable since it indicates a departure from the ideal model fit. The link among the mobile phase ratio (A), pH (B), and flow rate (C) concerning retention duration may be better understood with the help of polynomial Equation 1, 3D plots, and contour plots. This relationship is shown in figure 14. The retention time of xanthotoxin is significantly affected by the mobile phase ratio. A drop in the mobile phase concentration significantly rises the retention time of xanthotoxin, whereas an increase in the quantity of the mobile phase (ACN) ratio significantly decreases the time retention of xanthotoxin. Furthermore, longer retention durations are correlated with mobile phase ratios in the center.

The given polynomial equation for retention time (R1) is expressed as:

Equation 1

$$Retention\ time = +5.78 - 0.7125A + 0.6250B - 0.7125C + 0.0750AB + 0.8000AC + 0.1250BC - 0.5650A^2 + 1.21B^2 + 0.8850C^2$$

The equation illustrates the relationship between the independent variables, which include the xanthotoxin retention length, mobile phase ratio, pH, and flow rate. Reading

the equation's coefficients will show you how the retention time is affected by each variable and how they interact with one another. A negative effect, as indicated by coefficients such as -0.7125A, -0.7125C, and -0.5650A^2, indicates that retention time decreases as the related factors increase. Positive coefficients (e.g., 0.6250B, 0.0750AB, 0.8000AC) indicate that the associated variable has an influence that increases the retention time, indicating a positive effect. It is possible that the relationship is not entirely linear because of the presence of quadratic factors (A^2, B^2, C^2) suggests that the relationship may not be strictly linear, and the combined effect of the variables plays a crucial role in influencing the retention time.

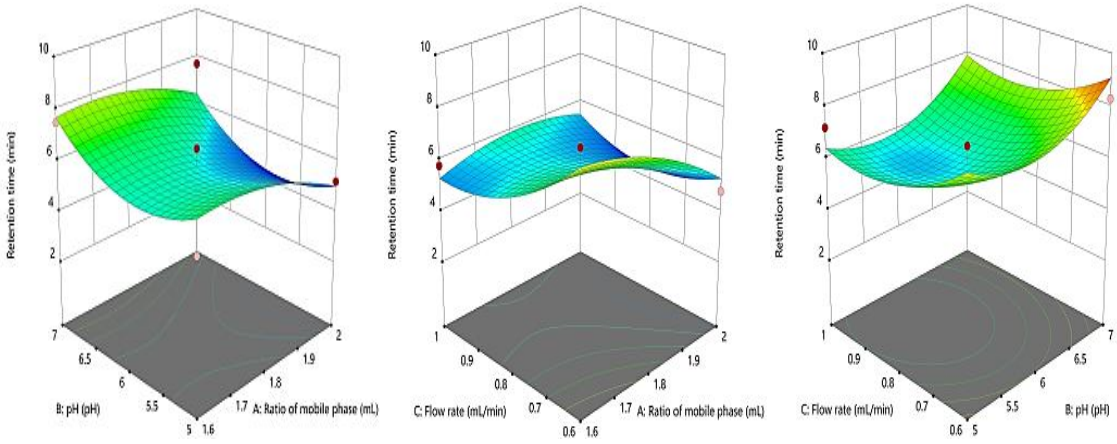


Figure 14: 3D response surface plots offer how the retention time (R1) of Xanthotoxin is influenced by the Mobile phase ratio (A), pH (B), and Flow rate (C).

5.2.2.4.2 Influence of independent variables on peak area (R2)

A Model F-value of 7.22 indicates that the model is statistically significant, suggesting that the factors included in the model help to explain the response variation. With such a big F-value and a low probability of 0.43%, the model is even more resistant to noise. The p-values for model terms A and B are less than 0.0500, indicating that they are significant. This highlights their importance in explaining variances in the response. Model reduction, removing few inconsequential terms (not including those needed to

support hierarchy), may improve overall performance, as values above 0.10 for model terms would indicate insignificance. With an F-value of 0.84, the Lack of Fit does not appear to be statistically significant when compared to the pure error. This Lack of Fit F-value could be attributable to noise 62.17% of the time, therefore a non-significant result is good because it means the model fits well. The goal of getting a model that fits the data well and shows all the patterns is met by doing this. Statistical metrics provide important information about the model's importance, individual terms' relevance, and overall fit. Table 26 shows how these criteria are used to inform judgments about improving and reducing models for better performance. Figure 15 shows the link between the mobile phase ratio (A), pH (B), and flow rate (C) on peak area, as shown in a polynomial equation 2 and three-dimensional and contour plots. The linear polynomial equation peak Area (R2) is represented as follows:

Equation 2

$$Peak\ Area = +1.903 \times 10^{05} - 31601.62A + 17817.88B - 8147C$$

This equation encapsulates the relationship between the factors (A, B, and C) and the peak area, providing a quantitative understanding of their influence on chromatographic performance.

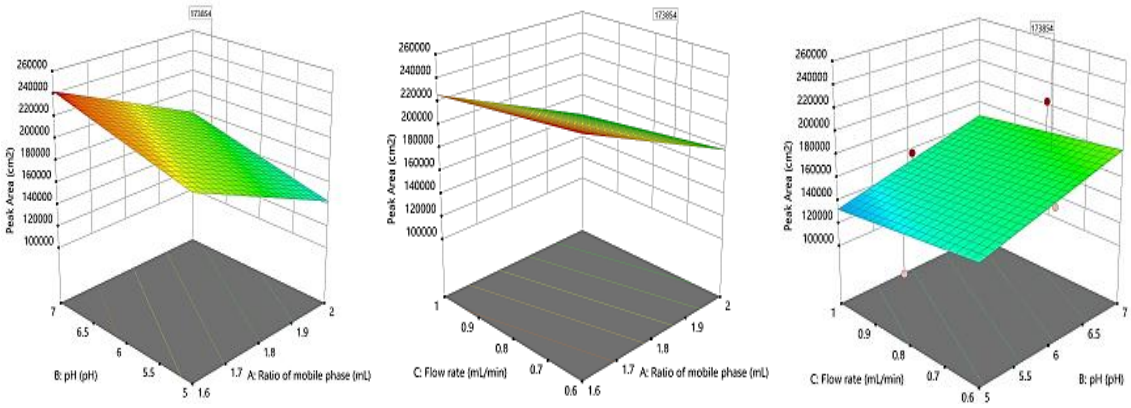


Figure 15: 3D response surface plots offer how the peak area (R2) of Xanthotoxin are influenced by the Mobile phase ratio (A), pH (B), and Flow rate (C).

5.2.2.4.3 Influence of independent variables on peak height (R3)

It is highly unlikely that such a large F-value could have happened by chance alone; the Model F-value of 5.38 proves that the model is significant. Variables C and AB are clearly significant contributors to the model, as their p-values are less than 0.0500, which is the threshold for determining the importance of model terms. On the other hand, a Miss Fit F-value of 0.81 indicates that the Miss Fit is not statistically significant when compared to pure error; a Miss Fit F-value of 0.81 may be explained by random variation by 61.17% of the time. As a sign of a good fit, a no significant Lack of Fit is ideal. A p-value greater than 0.1000 indicates insignificance, hence it is crucial to highlight the importance of particular model variables. To improve the overall effectiveness of a model, it is recommended to consider reducing the number of irrelevant words in it, excluding those required for hierarchy. These findings, as shown in Table 27, shed light on the importance of the model and the parts' contributions, which can help with future optimization efforts. As shown in Figure 16, the affiliation among the mobile phase ratio (A), pH (B), and flow rate (C) on peak area has been highlighted in the Equation 3, 3D plots, and contour plots. The given polynomial equation for Peak Height (R3) is expressed as follows:

Equation 3

$$Peak\ Height = +14000.94 + 34.38A - 53.62B + 600.00C + 1756.00AB - 537.75AC - 527.75BC$$

We can see how the three independent variables—A, B, and C—are interdependent on Peak Height in the given equation. Coefficients indicate the size and direction of influence and are thus significant indicators. Particularly, when all variables are at their reference levels, the intercept term of +14000.94 sets the baseline for Peak Height. Now we can see that a rise in the Mobile Phase Ratio (A) leads to a positive effect on Peak Height (coefficient = 34.38), and that a decrease in Peak Height (coefficient = -53.62) is indicated by an increase in pH (coefficient = -53.62). Alternatively, it can be inferred from the positive coefficient for C (+600.00) that an increase in Flow Rate (C) positively

affects Peak Height. In addition, the positive coefficient for AB (1756.00) suggests that Mobile Phase Ratio (A) and pH (B) combine to collaboratively boost Peak Height, as shown by the interaction terms (AB, AC, BC), which provide insight into the synergistic effects of the individual variables.

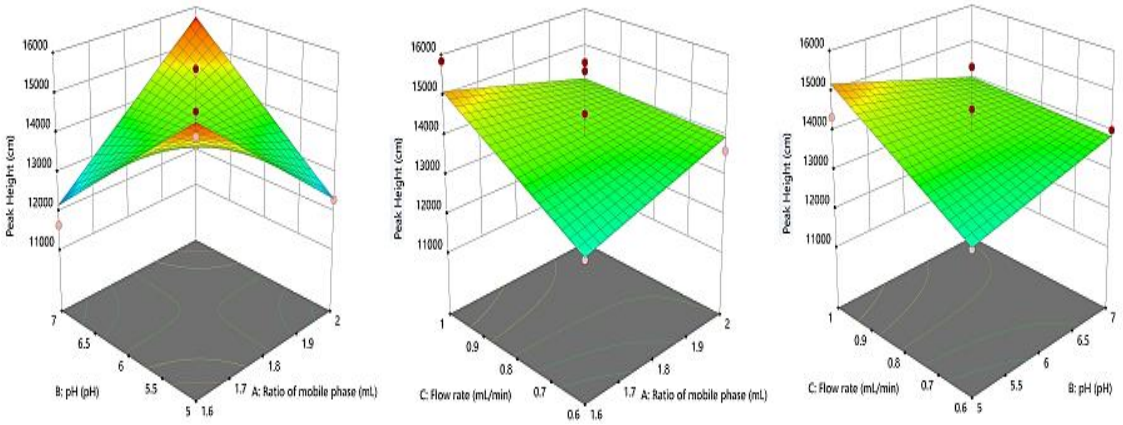


Figure 16: 3D response surface plots offer how the peak height (R3) of Xanthotoxin are influenced by the Mobile phase ratio (A), pH (B), and Flow rate (C).

Essentially, this all-encompassing analysis sheds light on the complex processes that influence the Peak Height response within the parameters provided.

5.2.2.4.4 Influence of independent variables on tailing factor (R4)

The Model F-value of 5.01 indicates that the overall model is statistically significant, suggesting that the included variables collectively contribute to explaining the variation in the response. The associated low probability (2.26%) of obtaining an F-value of this magnitude by random chance reinforces the model's significance. Examining individual model terms, A^2 stands out as statistically significant ($p < 0.050$), underscoring its relevance in explaining variations in the response. It is crucial to note that values exceeding 0.1000 for model terms would imply insignificance. Additionally, the Lack of Fit F-value, at 44.56, points to a significant lack of fit. This low probability (0.16%) suggests that the lack of fit likely not due to random noise. A substantial lack of fit is undesirable, emphasizing the importance of refining the model for better predictive accuracy. If there are numerous insignificant model terms, excluding them might enhance the model's overall performance. Overall, these statistical measures provide valuable

insights for model assessment and potential refinement, as depicted in table 27. The association between the mobile phase ratio (A), pH (B), and flow rate (C) on peak area has been established using polynomial Equation 4, 3D graphs, and contour plots as illustrated in figure 17.

The provided equation for the tailing factor (R4) is as follows:

Equation 4

$$Tailing\ Factor = +1.12 - 0.0132A - 0.0176B - 0.0324C - 0.0218AB + 0.0258AC - 0.0500BC - 0.1091A^2 + 0.0352B^2 + 0.0247C^2$$

The equation provides insights into the individual and combined effects of factors A (ratio of mobile phase), B (pH), and C (flow rate) on the Tailing Factor. The constant term (1.12) represents the baseline Tailing Factor at reference levels. Negative coefficients for some factors (-0.0132A, -0.0176B, -0.0324C) suggest their individual negative influence. Quadratic terms (-0.1091A^2, 0.0352B^2, 0.0247C^2) indicate nonlinear relationships. Interaction terms (-0.0218AB, 0.0258AC, -0.0500BC) reveal how pairs of factors jointly influence the Tailing Factor. Overall, increasing levels of A, B, and C tend to decrease the Tailing Factor, with quadratic and interaction terms adding complexity. All terms, including quadratic and interaction terms, are deemed significant, emphasizing the model's validity. This understanding is crucial for method development, aiming to minimize the Tailing Factor for accurate and reliable chromatographic results.

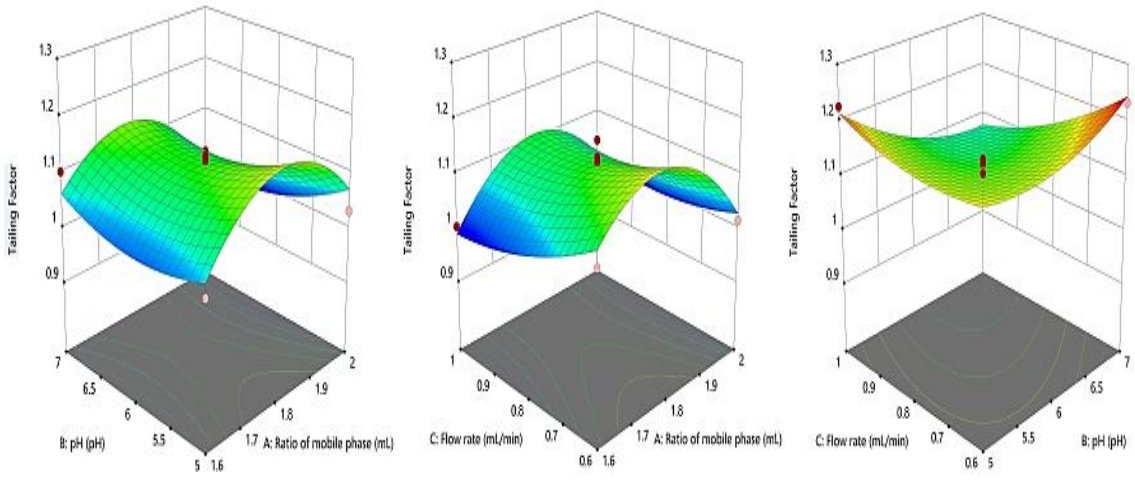


Figure 17: 3D response surface plots offer how the tailing factor (R4) of Xanthotoxin are influenced by the Mobile phase ratio (A), pH (B), and Flow rate (C).

5.2.2.4.5 Influence of independent variables on theoretical plate (R5)

While there is only a 0.05% chance of such a huge F-value happening owing to noise, the Model F-value of 11.67 strongly implies the statistical importance of the model, reinforcing its trustworthiness. With a p-value less than 0.0500, model terms are considered significant. In this case, term A stands out as statistically significant, highlighting its importance in explaining response variances.If the value is more than 0.1000, however, it indicates that the model terms are not significant. Excluding terms required for hierarchy, model reduction could improve overall model performance if there are several of these words. A significance level of 1.42 for the Lack of Fit F-value suggests that it is not statistically significant when compared to pure error. With a 39.17% chance of a Lack of Fit F-value this large occurring due to noise, the non-significant lack of fit is favorable, indicating a good fit for the model and contributing to a more accurate representation of the data, as depicted in table 27. Figure 18 shows the link between the mobile phase ratio (A), pH (B), and flow rate (C) on peak area, as seen in the polynomial Equation 5, 3D plots, and contour plots.The linear polynomial equation for theoretical plates (R5) is represented as follows:

Equation 5

$$Peak\ Area = +1.903 \times 10^{05} - 31601.62A + 17817.88B - 8147C$$

This equation encapsulates the correlation between the factors (A, B, and C) and the theoretical plates, providing a quantitative understanding of their influence on the chromatographic performance.

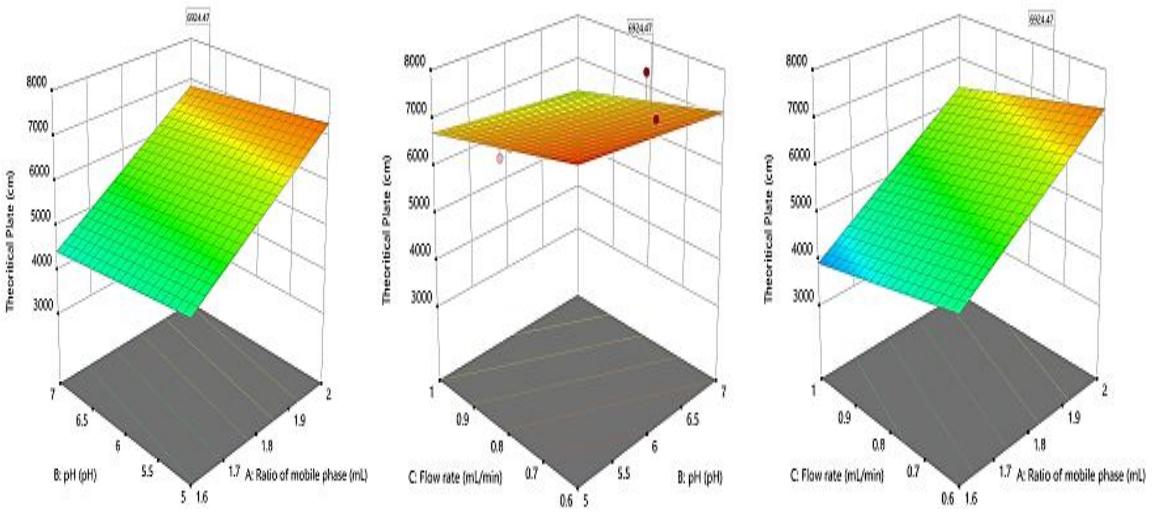


Figure 18: 3D response surface plots offer how the theoretical plate (R5) of Xanthotoxin are influenced by the Mobile phase ratio (A), pH (B), and Flow rate (C).

5.2.2.5 Enhancement of chromatographic methodology

Numerical optimization methods were harnessed to discern the optimal chromatographic conditions, a process involving the systematic manipulation of various independent variables. The objective was to achieve favorable outcomes, including the largest possible area under the peak and the highest number of theoretical plates, while also minimizing the time it takes for a compound to elute and the extent of tailing in the chromatographic separation. The pinnacle solution materialized through the elevation of mobile phase ratio (ACN: water) and pH to higher levels, coupled with a reduction in flow rate to 0.8 ml/min. Projections for the optimized chromatographic conditions yielded a retention time of 5.6094 min, peak area reaching 173,862 mAU, peak height of 15,315.4 AU, a tailing factor of 1.00307, and an impressive theoretical plate count of 6,924,018 cm. The resultant desirability value, a commendable 0.773, underscored the success of the optimization process. Furthermore, a comprehensive approach was employed, integrating graphical optimization techniques to delineate the optimal analytical design space. Visual representations in Figures 19-21 elucidate the refined solution, enhancing our understanding of the interplay among variables and pinpointing

the precise conditions that yield superior chromatographic performance. This multifaceted optimization strategy not only enhances the efficiency of the chromatographic process but also contributes to a deeper comprehension of the intricate relationships between parameters in the analytical domain.

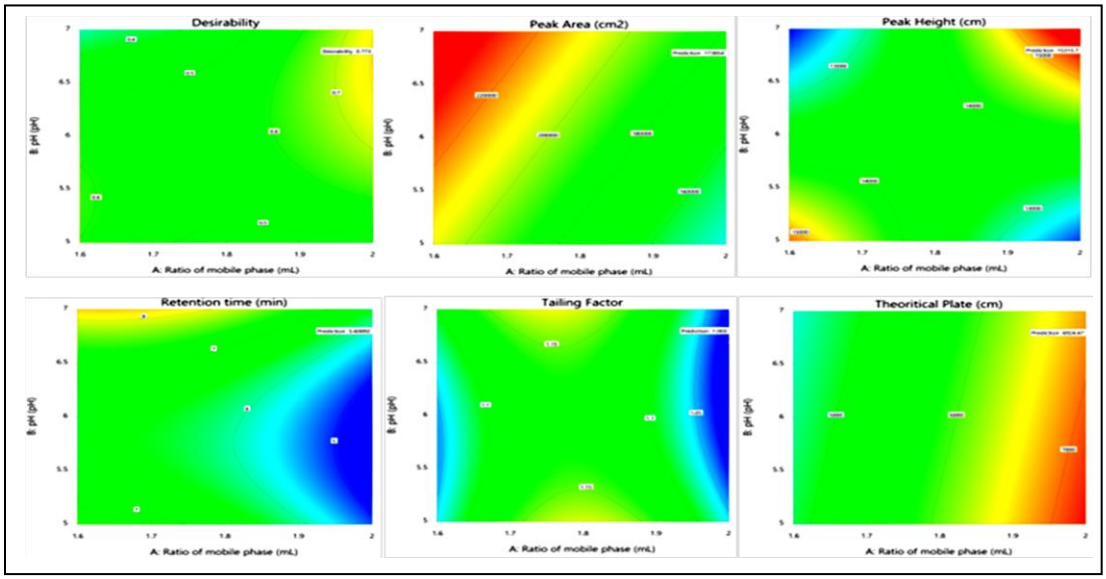


Figure 19: The influence of the mobile phase ratio (A) and pH (B) on optimized 2D-contour plots is examined, illustrating the deal solutions and appropriate dependent variables: Retention time (R1), Peak area (R2), Peak height (R3), Tailing factor (R4), and Theoretical plates (R5).

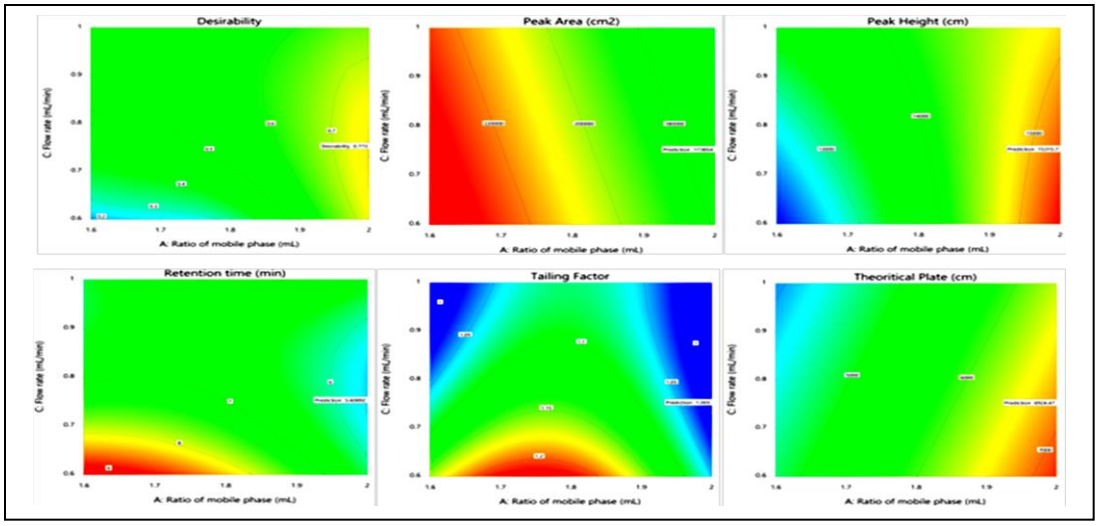


Figure 20: The effect of the mobile phase ratio (A) and flow rate (C) on optimized 2D-contour plots is examined, illustrating the optimal solutions and desirability of dependent variables: Retention time (R1), Peak area (R2), Peak height (R3), Tailing factor (R4), and Theoretical plates (R5).

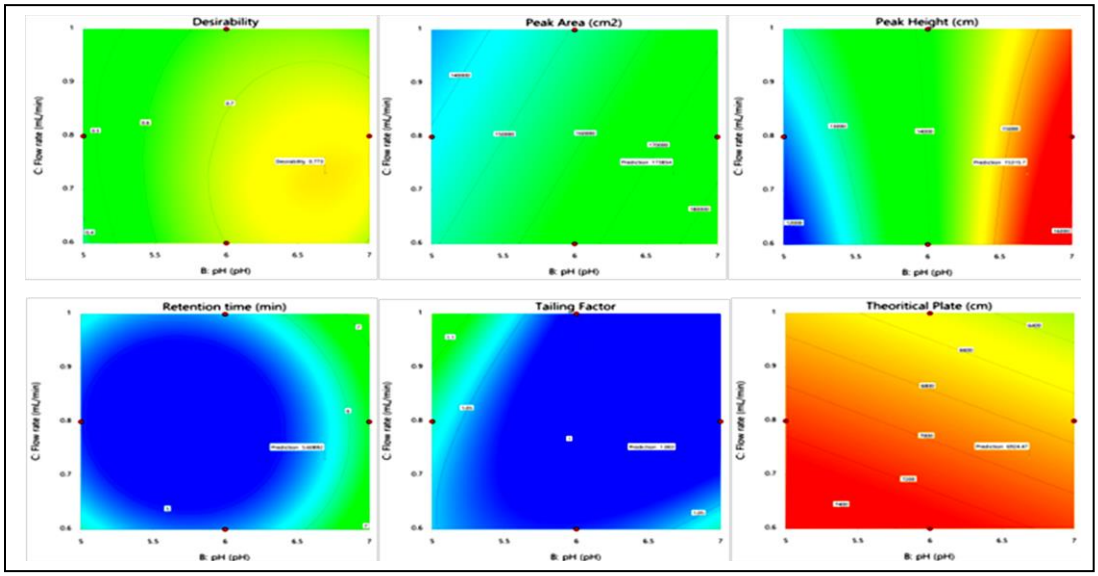


Figure 21: The effect of pH (B) and flow rate (C) on optimized 2D-contour plots is examined, illustrating the optimal solutions and desirability of dependent variables: Retention time (R1), Peak area (R2), Peak height (R3), Tailing factor (R4), and Theoretical plates (R5).

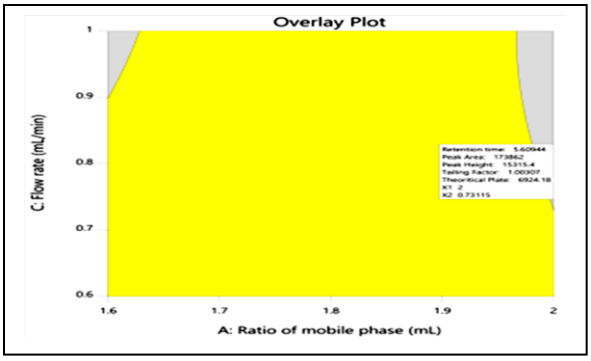


Figure 22: Overlay plot of flow rate vs. mobile phase ratio

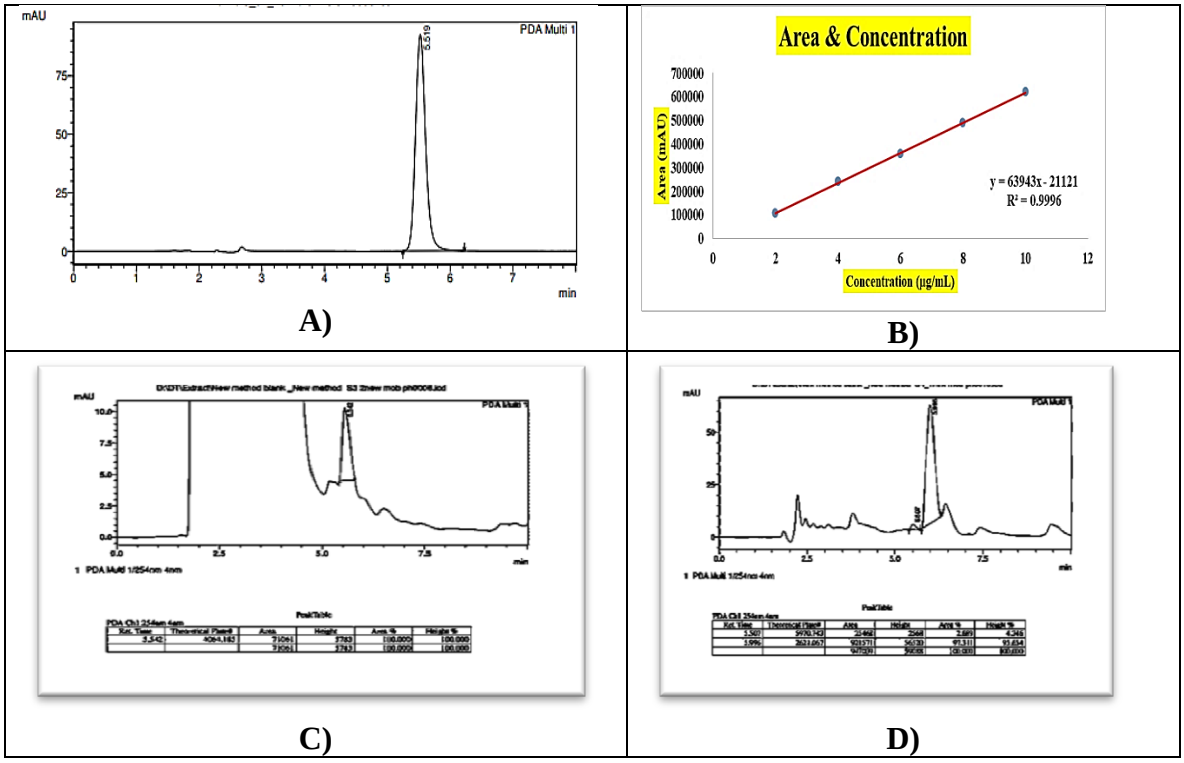


Figure 23: HPLC Chromatograms of various samples: A) =Chromatogram of xanthotoxin; B) = Calibration curve for xanthotoxin; C) = Chromatogram of chloroform extract; D) = Chromatogram of ethyl acetate extract.

5.2.2.6 Method validation

5.2.2.6.1 Linearity and range

The calibration curve for xanthotoxin spanning 2–10 µg/ml demonstrates excellent linearity, as depicted in Figure 23. The curve exhibits a commendable regression coefficient (r^2) of 0.9996, affirming the reliability of the analytical method and its

precision in accurately determining xanthotoxin concentrations within the specified range.

5.2.2.6.2 LOD and LOQ

The LOD and LOQ values, recorded at 0.58 μg/ml and 1.758 μg/ml, respectively, underscore the capability of the proposed method to discern low concentrations of xanthotoxin with precision and reliability.

5.2.2.6.3 Precision

Three control samples of xanthotoxin were tested at LQC, MQC, and HQC, respectively. The % RSD values were determined in quintuplicate for all studied concentrations, as presented in Table 28, exhibited consistently rang for intraday (0.283–0.565%), inter-analyst (0.593–0.748%), and interday (0.465–0.657%) precision was consistently below 2%, indicating that the results fell within acceptable limits. This observation attests to the precision of the developed method, affirming its reliability in producing consistent and accurate results.

Table 28: Displays the precision data that has been represented

Standard levels	Concentration (µg/ml)	Precision Parameters					
		Intraday		Inter-Analyst		Interday	
		(Mean Area ± SD) (n = 5)	%RSD	(Mean Area ± SD) (n = 5)	%RSD	(Mean Area ± SD) (n = 5)	%RSD
LQC	4.8	274610.2±1551.60	0.565	-		262135.6±1429.9	0.544
				A1 354513± 2650.7	0.748		
MQC	6	355713 ± 2253.09	0.633	A2 348259± 1962.3	0.563	346158.7±2271.6	0.657
				A3 343984± 2236.5	0.650		
HQC	7.2	429616 ± 1216.29	0.283	-		430669.7±2002.8	0.465

5.2.2.6.4 Accuracy

The accuracy assessment of xanthotoxin was conducted by calculating % recovery for three distinct concentrations, as delineated in Section 3.3. The % recovery exhibited a range from 95.86% to 97.84%, indicative of a satisfactory level of accuracy. Furthermore, the % RSD values, all below two, substantiated the precision and reliability of the results. Consequently, the detailed findings of the accuracy study are presented in Table 29.

Table 29: Displays the accuracy data that has been represented

Standard levels	**Concentration of Standard (µg/ml)**	**Concentration of Sample (µg/ml)**	**Accuracy Parameters**			
			Area (n = 5)		Sample (n = 5)	
			Mean ± SD	%RSD	% Recovery Mean ± SD	% RSD
LQC	4.8	4.610512488	273689 ± 921.44	0.337	96.05 ± 0.30	0.312
MQC	6	5.870221916	354238.6 ± 1231.23	0.348	97.84 ± 0.32	0.328
HQC	7.2	6.901771891	420199 ± 1080.18	0.257	95.86 ± 0.23	0.245

5.2.2.6.5 Robustness

The method's robustness was evaluated by varying parameters such as wavelength (250, 254, and 258 nm) and flow rate (0.6, 0.8, 1.0 ml/min). The % RSD values obtained were consistently less than 2%, signifying the method's robust performance under these deliberate variations. The detailed results of the robustness study are presented in Table 30.

Table 30: Displays the robustness data that has been represented

Variables	**Value**	**Concentration (µg/ml)**	**Robustness Parameters**			
			Area		Retention time (Rt)	
			(Mean Area ± SD) (n = 6)	%RSD	(Mean Rt± SD) (n = 6)	%RSD
Flow rate (ml/min)	0.6	6	361091.67±721.92	0.20	5.56±0.02	0.39
	0.8	6	355865.83±1046.64	0.29	5.53±0.07	1.28
	1	6	348459.33±715.76	0.20	5.52±0.09	1.63
Wavelength (nm)	250	6	344000.83±975.08	0.28	5.89±0.10	1.71
	254	6	356820.33±1777.23	0.50	5.96±0.10	1.69
	258	6	348971.33±1135.59	0.32	5.77±0.10	1.75

5.2.2.6.6 Factors for system compatibility

After five injections of a 10 μg/ml solution, the system suitability assessment revealed an absence of significant alterations in critical attributes, encompassing retention time, theoretical plates, peak area, and HETP of xanthotoxin. The system suitability parameters are presented in Table 31.

Table 31: Displays the system suitability parameters

Parameter	Value	Limit
Retention time	5.542	Depends on column length
Theoretical plates	5675.413	>2000
Peak area	622053	Depends on concentration
HETP	64545	Depends on theoretical plate

5.2.3 Conclusion

When it comes to quantitative analysis of polyphenolic compounds, HPLC is the way to go due to its adaptability, accuracy, and affordability (Govindarajan *et al*., 2007). In order to find a solvent that extracts the most of the chemicals studied, the extraction parameters for *H. candicans* were fine-tuned during the development of the process. In addition, according to García-Campaña *et al*. (2000), the method that was created was checked for selectivity, linearity, precision, accuracy, and robustness using the ICH guidelines (García-Campaña *et al*., 2000). Some of the furocoumarins found in the plant are 8-gernoxypsoralen (Y. N. Sharma *et al*., 1966), bergapten (Nath, 1961), heraclenol, xanthotoxol, phellopterin, imperatorin, angelicin, xanthotoxin (Bandopadhyay, 1973), candibirin (Doi *et al*., 2004), and heraclenin (Y. N. Sharma *et al*., 1964). Heraclenin is the anti-inflammatory ingredient found in *H. candicans*, according to activity-guided isolation (Bal-Tenbe, 1996). L-phenylalanine is changed into polyphenolic furocoumarins. L-phenylalanine is found in a straight line, having furan ring connected to the 6, 7 position of the benzo-2-pyrene nucleus. Many plant species, especially those in the Rutaceae and Umbelliferae families, contain these naturally occurring chemicals, which have biological activities (Guo & Yamazoe, 2004; Wagstaff, 1991). As previously noted by Ashwood-Smith *et al*. (1980), these substances are known to cause photo dermatitis when exposed to ultraviolet radiation (Ashwood-Smith *et al*., 1980).

The management of psoriasis in both hominids and experimental animals involves the use of UV radiation in conjunction with chronic furocoumarin treatment (Baumgart *et al*., 2005). Additionally, furocoumarins can covalently modify proteins, which can lead to the creation of new antigens (Schmitt *et al*., 1995). Because of their photosensitizing effects on human skin, furocoumarins from *H. candicans* find widespread application in pharmaceuticals (Devagiri *et al*., 1996).

Studies that used HPLC-PDA with a basic RP-18 column (Govindarajan *et al*., 2007; D. P. Singh *et al*., 2007) brought attention to the prominence of furocoumarins and *H. candicans*. The separation and resolution efficiency of the HPLC column dictate marker quantification, making it an essential component of every HPLC analysis. How well and how quickly substances can be separated using high-performance liquid chromatography (HPLC) depends on the type of RP column used, which in turn is determined by the length of the carbon chain or the ligand chemistry of the column When deciding on an analytical protocol, it is important to weigh the time and money spent on it against the separation efficiency.

6.1 *In vitro* anti-oxidant activity of extracts

In recent years, a growing body of clinical research has provided increasing substantiation for the immune theory (Rork *et al*., 2016; Rosmarin *et al*., 2020; Y. Wang *et al*., 2019). The depletion of melanocytes and the inhibition of melanin biosynthesis represent causative factors in the pathogenesis of vitiligo (Namazi, 2007; Ralf Paus *et al*., 2008). Melanin synthesis occurs within melanocytes in the course of a biological progression known as melanogenesis, which is facilitated by the enzymatic activities of Tyrosinase associated protein-1 and Tyrosinase associated protein-2. Notably, melanocytes derived from unaffected vitiligo skin exhibit reduced growth efficiency compared to those obtained from individuals with unimpaired skin. This diminished proliferation and function are accompanied by observable structural anomalies, indicative of inherent cellular abnormalities (Boissy *et al*., 1991; Puri *et al*., 1989). In comparison to normal melanocytes, they exhibit an increased susceptibility to exogenous oxidative stressors, leading to a higher rate of mortality (Jimbow *et al*., 2001; Maresca *et al*., 1997). These entities exhibit heightened quantities of reactive oxygen moieties (Schallreuter *et al*., 1999a; Shalbaf *et al*., 2008) and greater endoplasmic reticulum stress (Boissy *et al*., 1991). Incontrovertible empirical data substantiates that this stress signifies a pivotal part in the onset and evolution of pathological conditions (Denat *et al*., 2014). In the context of depigmentation and the progression of vitiligo, cellular oxidation stress serves a significant function in the beginning stage. Subsequent undesirable autoimmune responses then attributes a pivotal role in driving the evolution of the condition (Denman *et al*., 2008; Harris, 2016; Kroll *et al*., 2005; Laddha *et al*., 2013; Mosenson *et al*., 2013). Many extrinsic and endogenous factors have been linked to the genesis of a number of different illnesses. Substantial contributors to disease etiology comprise intrinsic variables such as melanin synthesis, cellular proliferation, differentiation, metabolic processes, immunological responses, and apoptosis (Al-Shobaili & Rasheed, 2015).

Extrinsic stimuli encompass various factors originating from the external environment, including but not limited to carcinogenic substances, physical injury, and radiation with UV light, monophenones, or similar phenolic chemical substances. Additionally, these stimuli may also arise from other disease states such as severe infections, neurological disorders, malignancies, and disturbances in calcium homeostasis. Furthermore, pharmaceutical interventions, such as specific hormones, vaccination procedures, and various drugs, can also serve as exogenous stimuli (Xie *et al*., 2016). All of these factors elicit oxidative stress within melanocytes, a phenomenon of potential significance in the initiation of autoimmune responses linked to the vitiligo pathogenesis (Abdel-Malek *et al*., 2020; Chen *et al*., 2019; Xie *et al*., 2016). Reduced levels and effectiveness of antioxidative enzymes along with the disruption in the balance involving pro-oxidation and anti-oxidation, are contributing variables in the accumulation and development of harmful oxygen radicals (Wang *et al*., 2019). The oxidative distress in the cells can induce the initiation of stress signaling pathways and the transcriptional variables that are coupled with them (Schallreuter *et al*., 2004; Shajil *et al*., 2006).

6.1.1 Experimental of anti-oxidant activity

6.1.1.1 Materials and methods

6.1.1.1.1 Chemicals

Chemicals utilized during the study includes: Folin–Ciocalteau's reagent, sodium carbonate, gallic acid, $AlCl_3$, methanol, quercetin, DPPH, phosphate buffer, potassium ferricyanide, trichloroacetic acid, ascorbic acid, linoleic acid, ferric nitrate, hydrogen peroxide, thiobarbituric acid. All these chemicals were of analytical grade and were purchased from Sigma Laboratories, CDH Laboratories, Merck.

6.1.1.1.2 Instruments

Instruments used included- UV-Visible spectrophotometer (Model U-2900, Hitachi, Tokyo Japan); UV-Visible spectrophotometer (Model UV-2450, Shimadzu, Japan)

6.1.1.2 Total Phenolic content (TPC)

The Folin–Ciocalteau method reported by Cindric *et al*. (2011) was used to assess total phenolic content with minimal changes. 2 g of pulverized plant specimens were refluxed using 50 ml of corresponding extraction solvents for 2 hrs. The residue was filtered, refluxed for 1 hr, and then filtered again. In each Eppendorf tube, 5 ml of Folin–Ciocalteau's reagent was mixed with 0.2 ml of recovered filtrate and 0.8 ml of filtered water. Further, 4 ml of 20% sodium carbonate solution was added over 5 mins, and the mixture was incubated in the dark for 90 mins. Absorption spectra were recorded at 760 nm using a UV-visible spectrophotometer. Polyphenol content was measured in mg of gallic acid equivalence pergram (mg GAE/g) using the reference calibration (Juranović Cindrić *et al*., 2011).

6.1.1.3 Total flavonoid content (TFC)

It was assessed using the methodology described by Krishnaiah *et al*. in 2012, with minor adjustments. The dried extracts (100 mg) were reconstituted again in a solution of 15 ml of methanol and water (50% v/v). After mixing, the solution was centrifuged at 2000 rpm for 10 mins. An identical proportion (5 ml) of $AlCl_3$ solution (2% w/v in methanol) was poured to the supernatant. The absorption was recorded at a wavelength 415 nm against a blank using a UV-Vis spectrometer for a total runtime of 10 mins. A reference curve for quercetin ranging from 0 to 100 µg/ml was established, and the total flavonoid content was assessed in mg of quercetin equivalents per gm. (mg QE/g) (Krishnaiah *et al*., 2012).

6.1.1.4 DPPH radical-scavenging activity

It was analyzed employing a revised variant of the methodology reported by Brand-Williams *et al*. in 1995. After being dissolved in 10 ml of methanol for 2 hrs, a 100 mg sample was centrifuged at 3000 rpm for 10 mins. Collected the liquid above the solid surface (100 µl) and added 3.9 ml of DPPH solution ($6x10^5$ mol/l). The materials' transmittance (A) was quantified, utilizing a wavelength of 515 nm after 0 and 30 mins, with methanol acting as the blank (Brand-Williams *et al*., 1995). The estimation of anti-oxidant capacity was performed utilizing the preceding calculation:

$$DPPH\ radical\ scavenging\ activity\ (\%) = \left\{1 - \left[\frac{A\ of\ sample\ at\ t = 30}{A\ of\ sample\ at\ t = 0}\right]\right\} \times 100$$

Where A= absorbance of sample, t= time

6.1.1.5 Reducing Power (RP)

The extracted samples' reduction power was assessed using Liu *et al*. 2015 technique. In conclusion, freeze-dried materials were re-dissolved in the solvents of extraction at 15 mg/ml. Then, 2.5 ml of the specimen solutions were merged with 2.5 ml of phosphate buffer (0.2 M; pH 6.6) and potassium ferri-cyanide (10 mg/ml). The ingredients were properly blended and bathed in 50°C water for 20 min. After cooling to room temperature, 2.5 ml of 10% trichloroacetic acid (TCA) was applied. The resulting product was centrifuged at 3,000 rpm for 10 min. The optical density at 700 nm was measured after mixing the liquid using 0.5 ml of $FeCl_3$ solution (1 mg/ml). A typical curve made using ascorbic acid estimated the data in AAE/g of extract(H. Liu *et al*., 2015).

6.1.1.6 Lipid peroxidation inhibition assay (% ILP)

H. candicans extract ILP values were assessed employing Wright *et al*.'s 1981 modified approach. The 200 mg freeze-dried specimens have been reconstituted in their solvents. To the 250 µl supernatant layer, 1 ml linoleic acid (0.1%), 0.2 ml ascorbic acid (200 mM), 0.2 ml ferric nitrate (20 mM), and 0.2 ml hydrogen peroxide was introduced. TCA (10% w/v) and TBA (1 ml, 1% w/v) were added to the mixture and heated in a water bath at 37°C for 1 hr until the reaction is stopped. After 20 min of heating in water, the resultant mixture was centrifuged at 5000 rpm for 1 min and absorbance was recorded at 350 nm using the UV spectrophotometer. The % inhibition of lipid peroxidation was established using this equation:

$$\%\ \text{Inhibition} = [1 - (A\ \text{of sample}/A_0\ \text{of control})] \times 100$$

Where A= absorbance of sample and A_0 = absorbance of control/standard.

6.1.2 Results of the anti-oxidant studies

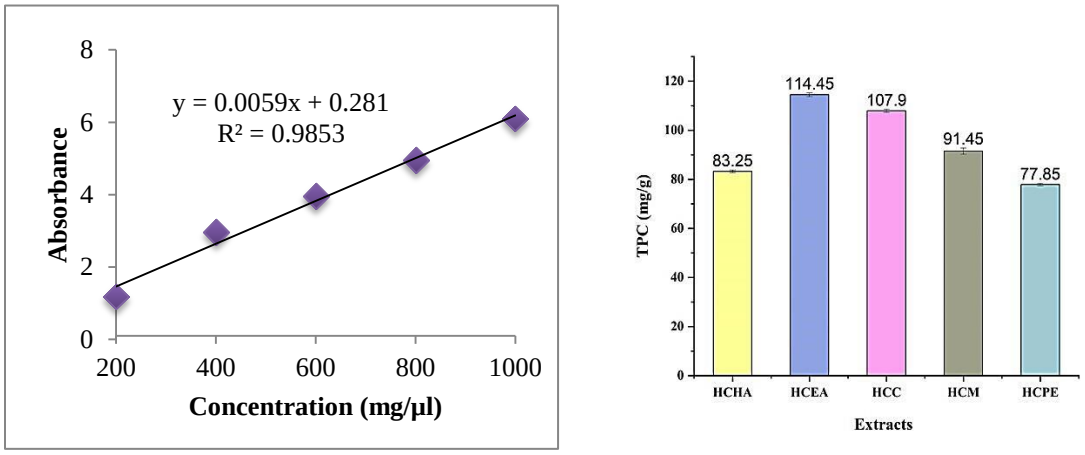
6.1.2.1 Total Phenolic content

The anti-oxidant power of botanicals is primarily associated with the presence of phenolic compounds and flavonoids. Polyphenols as natural anti-oxidants found in various food sources, play a significant role in these pharmacological activities. While their anti-oxidant capacity accounts for their observed effects, beyond the potential lowering of oxidative stress, its biological consequences may be far-reaching (Scalbert & Williamson, 2000). The results are expressed in mg of GAE per gram of extract.

Table 32: Total phenolic content (mg/g)

Extracts	TPC
Petroleum ether extract	77.85±1.093[b]
Chloroform extract	107.9±0.92[b]
Ethyl acetate extract	114.45±0.76[a]
Methanolic extract	91.45±0.55[c]
Hydroalcoholic extract	83.25±0.50[d]

Where a, b, c, d indicate the significance of values reported during the evaluation on the basis of various P values.



(A) (B)

Figure 24: Total phenolic content (A= Calibration curve of TPC; B= Total phenolic content of various extracts); HCHA= *Heracleum candicans* hydroalcoholic, HCEA= *Heracleum candicans* ethyl acetate, HCC= *Heracleum candicans* chloroform, HCM = *Heracleum candicans* methanolic, HCPE= *Heracleum candicans* petroleum ether extract.

The results of TPC study revealed that ethyl acetate, chloroform, petroleum ether, methanolic, hydroalcoholic extracts provided 114.45, 107.9, 77.85, 91.45, 83.25 mg/g of total phenolic content, respectively. Results showed that ethyl acetate extract have the maximum TPC followed by chloroform extract. Whereas hydroalcoholic extracts have the minimum total phenolic content. The highest phenolic content in the ethyl acetate extract of the aerial part of the plant can be attributed to the presence of various phenolic and furanocoumarins compounds. However, the exact nature of compounds which may be responsible needs further studies like isolation and fractionations of the extracts using various chromatographic techniques. In a study conducted by Falleh *et al*. (2008), it was observed that the composition and quantity of phenolic compounds exhibit significant variability due to various intrinsic and extrinsic factors (Falleh, 2008). This variability consequently leads to variations in anti-oxidant activity, total phenolic content, and flavonoid concentrations among different cultivars. Furthermore, the process of extraction substantially influences the ultimate characteristics, composition, and content of the extracted material (Gallardo *et al*., 2006).

6.1.2.2 DPPH radical-scavenging activity of various extracts

In previous research, polyphenolics have been demonstrated to serve a significant role in augmenting the anti-oxidant ability of the diverse botanicals (Pawar *et al*., 2011). A diamagnetic molecule arises through the acquisition of hydrogen radical or an electron by DPPH which is stable. DPPH is typically employed as a substrate for the assessment of the anti-oxidant characteristics of various compounds present in plants. This scientific approach allows us to explore the prospective attributes of anti-oxidants in a responsible and compassionate manner.

Table 33: DPPH radical-scavenging activity of various extracts

Extracts	% DPPH
Petroleum ether extract	53.56 ± 0.32^{d}
Chloroform extract	85.21 ± 0.55^{a}
Ethyl acetate extract	74.78 ± 0.83^{b}
Methanolic extract	69.56 ± 0.95^{c}
Hydro alcoholic extract	64 ± 0.51^{c}

Where a, b, c, d indicate the significance of values reported during the evaluation on the basis of various P values.

Formula for calculating DPPH radical scavenging activity:

DPPH (%) = [1-(A of sample t = 30/A_0 of control t = 0)] × 100;

Where A = Absorbance of sample, A_0= absorbance of control/standard, and t= time

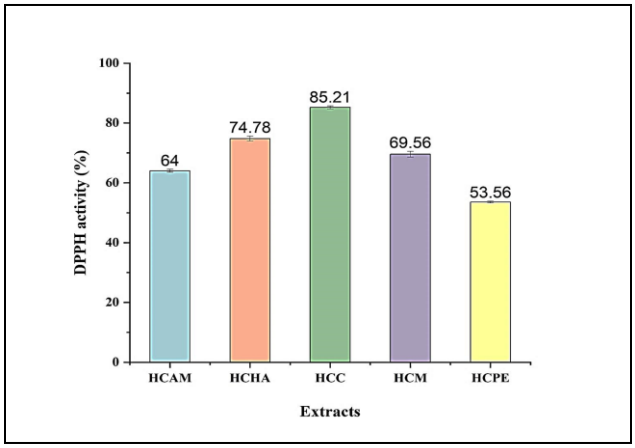


Figure 25: DPPH –radical scavenging activity of various extracts; HCAM= *Heracleum candicans* hydroalcoholic, HCHA= *Heracleum candicans* ethyl acetate, HCC= *Heracleum candicans* chloroform, HCM = *Heracleum candicans* methanolic, HCPE= *Heracleum candicans* petroleum ether extract.

The results of DPPH study revealed that ethyl acetate, chloroform, petroleum ether, methanolic, hydroalcoholic extracts provided 74.78, 85.21, 53.56, 69.56, 64% of DPPH radical scavenging activity, respectively. From the above results of DPPH radical scavenging activity, it can be concluded that the aerial chloroform extract of the plant indicated highest radical scavenging activity than the other extracts. Least scavenging activity by radicals was exhibited by the hydro alcoholic extract. Research has demonstrated an encouraging correlation involving the proportion of DPPH radicals scavenged and the potency of anti-oxidant potential (Sharif & Bennett, 2016).

6.1.2.3 Total Flavonoid Content (TFC) of various extracts in µg QE/ml

The quantification of TFC is denoted in milligrams of quercetin equivalents (or an alternative standard flavonoid) per gram of the specimen (µg QE/ml). Quercetin serves as a frequently employed reference standard in these assessments.

Table 34: Total flavonoid content of various extracts in µg QE/ml

Extracts	Total flavonoid content
Petroleum ether extract	27.8±1.04[d]
Chloroform extract	69.5±0.96[a]
Ethyl acetate extract	36.875±0.59[c]
Methanolic extract	48.8±0.50[b]
Hydro alcoholic extract	18.37±0.89[e]

Where a, b, c, d indicate the significance of values reported during the evaluation on the basis of various P values.

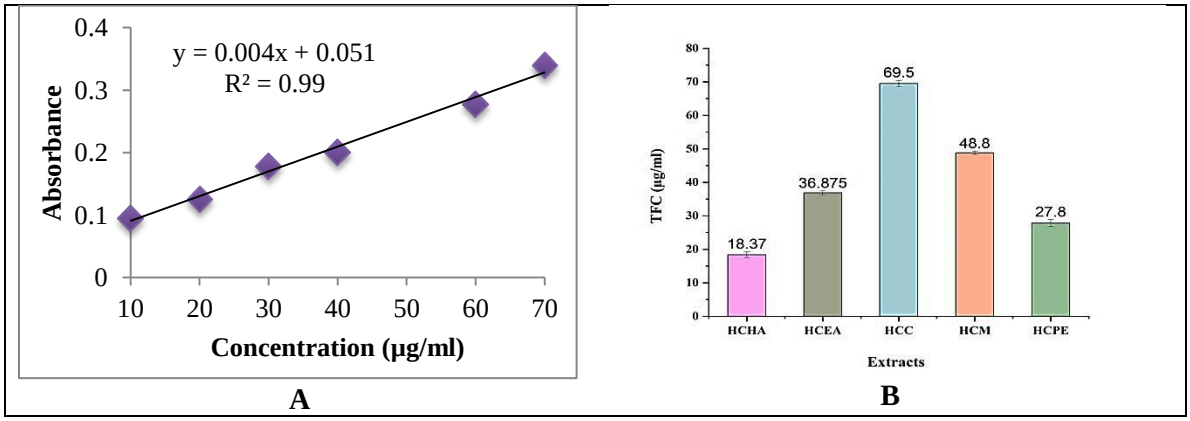


Figure 26: TFC of various extracts (A=Calibration curve; B=Total Flavonoid Content of various extracts); HCHA= *Heracleum candicans* hydroalcoholic, HCEA= *Heracleum candicans* ethyl acetate, HCC= *Heracleum candicans* chloroform, HCM = *Heracleum candicans* methanolic, HCPE= *Heracleum candicans* petroleum ether extract.

The results of TFC study revealed that ethyl acetate, chloroform, petroleum ether, methanolic, hydroalcoholic extracts provided 36.87, 69.5, 27.8, 48.8, 18.37 µg/ml of total flavonoid content, respectively. Results showed that chloroform extract have the maximum TFC followed by methanol and ethyl acetate extract. Whereas hydroalcoholic extracts have the minimum total flavonoid content of the aerial part of the plant.

6.1.2.4 Reducing power of various extracts

When polyphenolic compounds exhibit a pronounced capacity for reduction, it signifies their heightened anti-oxidant potential. This capacity is rooted in their ability to facilitate the conversion of the Fe_3^+/ferricyanide complex into its ferrous form (Ying Liu *et al*., 2015). The findings demonstrated a positive correlation between the content of extracted plants and the rate of absorption of the resulting solution. This suggests that the concentration of hydrogen donating components or the reducing power of the extracts was enhanced.

Table 35: Reducing power of various extracts in AAE/g

Extracts	Reducing Power
Petroleum ether extract	0.312 ± 0.011^{e}
Ethyl acetate extract	0.416 ± 0.045^{d}
Chloroform extract	0.680 ± 0.015^{b}
Methanolic extract	0.764 ± 0.023^{a}
Hydro alcoholic extract	0.530 ± 0.077^{c}

Where a, b, c, d indicate the significance of values reported during the evaluation on the basis of various P values.

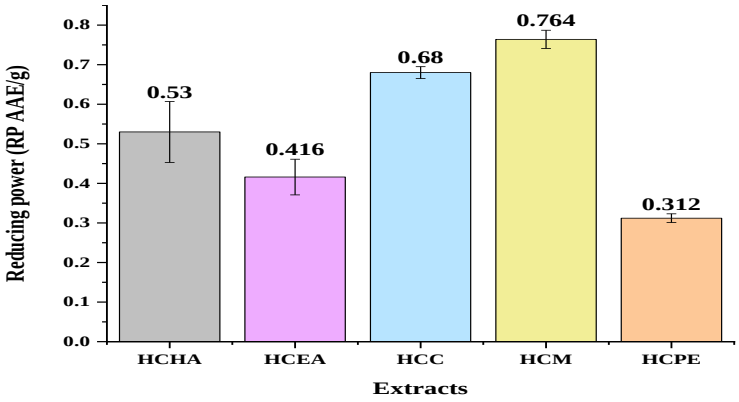


Figure 27: Reducing Power of various extracts; HCHA= *Heracleum candicans* hydroalcoholic, HCEA= *Heracleum candicans* ethyl acetate, HCC= *Heracleum candicans* chloroform, HCM = *Heracleum candicans* methanolic, HCPE= *Heracleum candicans* petroleum ether extract.

The results of reducing power study revealed that ethyl acetate, chloroform, petroleum ether, methanolic, hydroalcoholic extracts provided 0.416, 0.68, 0.312, 0.764, 0.53

AAE/g of reducing power, respectively. Results showed that methanol extract have the maximum RP followed by chloroform extract. Whereas petroleum ether extracts have the minimum total reducing power.

6.1.2.5 Lipid peroxidation inhibition

Table 36: Lipid peroxidation inhibition by various extracts

Extracts	ILP (%)
Petroleum ether extract	51.64±0.90[b]
Chloroform extract	55.87±0.68[b]
Ethyl acetate extract	68.17±0.39[a]
Methanolic extract	43.94±0.32[c]
Hydro alcoholic extract	34.50±0.46[d]

Where a, b, c, d indicate the significance of values reported during the evaluation on the basis of various P values.

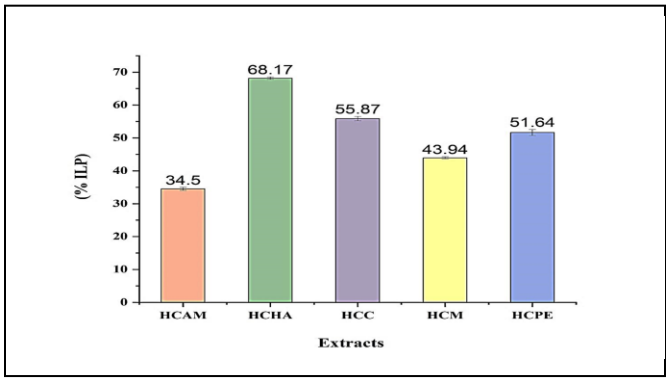


Figure 28: Lipid peroxidation inhibition of extracts; HCHA= *Heracleum candicans* hydroalcoholic, HCEA= *Heracleum candicans* ethyl acetate, HCC= *Heracleum candicans* chloroform, HCM = *Heracleum candicans* methanolic, HCPE= *Heracleum candicans* petroleum ether extract.

The results of the lipid peroxidation inhibition study revealed that ethyl acetate, chloroform, petroleum ether, methanolic, hydroalcoholic extracts provided 68.17, 55.87, 51.64, 43.94, 34.5 % ILP, respectively. Results showed that ethyl acetate extract have the maximum ILP (%) followed by chloroform extract, whereas hydroalcoholic extracts have the minimum lipid peroxidation inhibition.

6.1.3 Conclusion

In a recent research, findings have indicated a notable positive correlation between heightened lipid peroxidation concentrations and the occurrence of circulating anti-melanocyte antibodies in individuals diagnosed of vitiligo (Laddha *et al*., 2014). These free radicals are generated as secondary intermediaries during melanogenesis process, which is regulated by a multitude of anti-oxidative enzymes (Hensley *et al*., 2000; Maresca *et al*., 1997; Nordberg & Arnér, 2001). In relation to melanocyte destruction pathogenesis, free radical stress is regarded as the primary triggering event (Schallreuter, 1999). Vitiligo is concomitant with oxidative stress, marked by the excessive generation and accumulation of hydrogen peroxide and subsequent melanocyte degradation (Bowers *et al*., 1994; Knight, 1995; Yildirim *et al*., 2004). Moreover, in the correlation between reactive stress and autoimmunity, it has been hypothesized that oxidative stress potentially contributes causative function in initiation of vitiligo, whereas autoimmunity is implicated in the advancement of the pathological processes associated with the disease (Laddha *et al*., 2013, 2014). Superoxide dismutase constitutes a class of metallo-enzymes specialized in the catalysis of superoxide anion (O_2) dismutation during the detoxification process, resulting in the development of molecular oxygen and H_2O_2 (Koca *et al*., 2004). Individuals suffering with vitiligo have been demonstrated to exhibit increased amounts of superoxide dismutase in both vitiliginous and non-vitiliginous areas of the skin (Sravani *et al*., 2009). In our study, from the results obtained, it can be concluded that among the various extracts chloroform and ethyl acetate extract exhibit good anti-oxidant activity. While as least activity was seen in the petroleum ether extract.

6.2 *In vitro* anti-vitiligo activity of the extracts

Cell line studies were conducted to investigate the potential anti-vitiligo activities of various plant extracts (i.e. petroleum ether, chloroform, ethyl acetate, methanol and hydroalcoholic). These investigations aimed to explore the effects of the extracts on cell lines relevant to vitiligo, providing valuable insights into their therapeutic potential. The analysis encompassed a range of plant extracts, with the objective of discerning their

impact on cellular mechanisms associated with vitiligo, contributing valuable data to the ongoing exploration of novel treatment avenues for this skin disorder.

Tyrosinase, a versatile type-3 copper-containing glycoprotein anchored to the membrane, is situated within the melanosome membrane and is abundantly present in the natural environment (Sánchez-Ferrer *et al*., 1995). Tyrosinase, exclusive to melanocyte cells, undergoes processing being transported to melanosome for melanin synthesis. As a pivotal enzyme in melanogenesis, tyrosinase catalyzes the initial oxidations of L-tyrosine, converting it to 3,4-dihydroxy-L-phenylalanine (L-DOPA) and then to DOPA quinone. Given its critical role, various natural and synthetic inhibitors, including hydroquinone, ascorbic acid derivatives, azeleic acid, retinoids, arbutin, kojic acid, resveratrol, and polyphenolic compounds, have been identified for cosmetic and therapeutic applications (Arndt & Fitzpatrick, 1965; Fitzpatrick *et al*., 1966; Heilgemeir & Balda, 1981; Kligman & Willis, 1975). Numerous inhibitors of tyrosinase, such as, arbutin, kojic acid (Gonçalez *et al*., 2013; Ki *et al*., 2013; Kumar *et al*., 2013), azeleic acid (Breathnach *et al*., 1989; Verallo-Rowell *et al*., 1989), etc. and several tyrosinase inhibitors, like hydroquinone, have been employed as skin-brightening agents. This emphasizes the significance of identifying compounds that can reliably and securely modulate the activity of this enzyme. Tyrosinase activity was assessed by measuring the rate of L-DOPA oxidation. B16 cells were seeded in 6-well plates ($3x10^5$cells/well) and treated with test samples. After cell lysis and centrifugation, protein concentrations were determined using a BCA assay kit. Cell lysates (90 µl) were mixed with 10 µl of 10 mM L-DOPA in a 96-well plate and incubated at 37ºC in the dark. Dopa-chrome formation was measured at 490 nm to calculate tyrosinase activity for each sample.

MTT cytotoxicity studies were conducted on various extracts of the plant. These investigations sought to assess the impact of the plant extracts on cell viability, providing crucial insights into their safety profile. By employing the MTT assay, the studies aimed to elucidate the cytotoxic effects, if any, of the plant extracts, contributing valuable information for a comprehensive understanding of their biological effects.

6.2.1 Experimental

6.2.1.1 Materials and methods

6.2.1.1.1 Chemicals

The chemicals employed in the study included; DMSO, MTT reagent, FBS, antibiotic-antimycotic solutions,10 % FBS, B16F10-Mouse Skin Melanoma cell line (From NCCS, Pune), L-Tyrosine (Cat No: T3754, Sigma), Psoralen (Cat No:BD34569, BLD Pharma), Phenylmethanesulfonyl fluoride, D-PBS, α-melanocyte-stimulating hormone (α-MSH) (Cat No: 63605, Sigma), Sodium hydroxide (Himedia), Cell culture medium: DMEM-High glucose media - (Cat No:2120785, Gibco),

6.2.1.1.2 Instruments

Philips UV-A (9 W/10 amp), adjustable multichannel pipettes and a pipettor (Benchtop, USA), serological pipettes (TORSON), 96-well plate for culturing the cells (From Corning, USA), Centrifuge, Inverted biological microscope, 37°C incubator with humidified atmosphere of 5% CO_2 , 96-well plate ELISA reader

6.2.1.2 Cell viability studies

A cytotoxicity investigation will be undertaken employing the MTT reagent, according the standard procedure. B16F10 cells are sown at a density of 1×10^4 cells per well in a 96-well plate and incubated overnight. The cells are subjected to drug/sample solutions at concentrations ranging from 1-50 μg/ml. This is done to assess the IC_{50} level for each drug. After treatment, the cells are subjected to UV-A radiation for 15 mins via a Philips UV-A (9 W/10 amp), which emits light in the range of 315-400 nm at a maximum wavelength of 365 nm. This exposure occurs 1 hr after the treatment with the various extracts or formulations that are being tested. Following a 24 hr period, the MTT solution is introduced and the cells are subsequently maintained for duration of 3-4 hrs. Subsequently, DMSO is introduced and the resulting color is measured using a micro plate reader at a wavelength of 570 nm. The reference group consisted of untreated cells demonstrating 100% viability.

6.2.1.3 MTT Cytotoxicity studies

6.2.1.3.1 Maintenance of Cell Lines

The B16F10 (Mouse Skin Melanoma cell line) has been successfully utilized towards such aim due to its ease of *in vitro* cultivation and its resemblance with human melanocytes in terms of melanogenic processes (An *et al*., 2008). The cells are developed in DMEM higher glucose mix containing 10% FBS with 1% antibiotic-antimycotic solutions. The culture was kept in a CO_2 incubator under an ambiance of 5% CO_2 and 18-20% O_2 at a temperature of 37ºC. These cells were subsequently sub-cultured every 2 days.

6.2.1.4 Tyrosinase study

Melanin is formed by the melanocytes located in the basal layer of the epidermis. Its production is governed by an enzymatic cascade, which includes key players like tyrosinase, tyrosinase-related protein 1, and tyrosinase-related protein 2 (Sandoval-Cruz *et al*., 2011). Among these, tyrosinase plays a crucial role as the rate-limiting enzyme, orchestrating two vital processes that trigger melanin formation, comprise the hydroxylation of a certain monophenol along with the resulting transformation of an otho-diphenol to the equivalent ortho-quinone (Garcia-Molina *et al*., 2012; Niu *et al*., 2019).

6.2.1.4.1 Assay controls

(i) Medium control (medium without cells)

(ii) Negative control (medium with cells but without the experimental drug/compound)

(iii) Positive controls (medium with cells treated with Psoralen and Xanthotoxin and various extracts with concentrations ranging from 1-25 µg/ml)

6.2.1.4.2 Steps followed

1. Seed 1000 µl cell suspension in a 6-well plate at required cell density (50,000 cells per well), without the test agent, allowed the cells to grow for about 24 hrs.
2. Added appropriate concentrations of the test agent and incubate for 48 hrs.

3. After the incubation period, takeout the plates from incubator and removed spent media and washed the cells with 1x PBS twice and lysed the cells with 500 µl of lysis buffer (1% Triton X-100 prepared in PBS) at 4°C for 30 mins.
4. Centrifuged the lysed cell suspension at 14000 rpm for 15 mins and collected the supernatant.
5. For tyrosinase activity, made up the volume up to 100 µl with lysis buffer and then added 100 µl of 0.1% L-DOPA solution prepared freshly in PBS.
6. Transferred the whole contents into respective wells of 96-well plate and incubated at 37°C for 1 hr and measure the absorbance at 492 nm. The percentage tyrosinase activity is assessed using below equation:

Tyrosinase Activity = (absorbance of treated cells/absorbance of untreated cells) x 100.

6.2.1.5 Melanin estimation

Melanin estimation study aims to assess alterations in melanin content, elucidated helpful information about the prospective modulatory actions of the treatments on melanogenesis. Melasma, senile lentigo, freckles, and age spots are severe skin illnesses with abnormal and excessive melanin synthesis as their main cause. Melanocytes undergo melanogenesis when they are stimulated to produce melanin, a dark macromolecular pigment. The production of pigment is facilitated by an enzyme cascades and chemical reactions.

6.2.1.5.1 Steps followed

1. Seeded 1000 µl cell suspension in a 6-well plate at required cell density (50,000 cells per well), without the test agent, allowed the cells to grow for about 24 hrs.
2. Added appropriate concentrations of the test agent and incubated for 48 hrs.
3. After the incubation period, takeout the plates from incubator and removed spent media and wash the cells with 1x PBS twice and lyse the cells with 500 µl of lysis buffer (1% Triton X-100 prepared in PBS) at 4°C for 30 mins.

4. Centrifuged the lysed cell suspension at 14000 rpm for 15 mins and collected the pellet.
5. Cell pellet is dissolved using 1 N sodium hydroxide (NaOH) containing 10% DMSO for 1 hr at 80°C to solubilized the melanin.
6. Make up the volume up to 100 µl with NaOH solution and transferred the solution into 96-well flat bottom plate.
7. Melanin content in the samples was determined by measurement of the absorbance at 405 nm using 96-well plate reader.

   % Melanin content is calculated using below formula:

   % Melanin content=Abs of treated cells/Abs of Untreated cells x 100

6.2.2 Results of MTT assay using B16F10 cell line

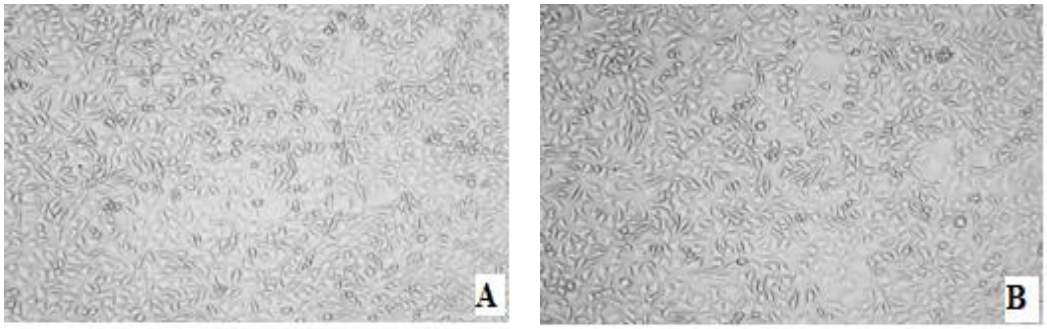


Figure 29: Cell viability studies A-B):A =Untreated cell line; B= Treated cellline with Chloroform Extract of the plant

Following a 24 hr incubation period, the results of the MTT assay conducted on the B16F10 cell line revealed critical insights into the cytotoxicity profile of the various plant extracts. The assay outcomes provide quantitative data on cell viability, shedding light on the potential impact of the extracts on the metabolic activity of the cells. The IC_{50} value was determined by using linear regression equation i.e. Y =mx+C. Where, Y = 50, m and C values were derived from the viability graph. The percentage viability of various treatment groups is shown in table 37.

Table 37: Percentage cell viability values of the extracts

Test group	Concentration (µg/ml)				
	1 µg	**5 µg**	**10 µg**	**25 µg**	**50 µg**
Psoralen	99.5822	98.2591	95.961	94.2201	83.844
Xanthotoxin	98.7465	95.6825	91.5042	83.3565	66.9916
Petroleum ether extract	96.0306	91.3649	86.4903	63.5097	27.7159
Chloroform extract	98.9554	94.6379	91.156	82.4513	67.2702
Ethyl acetate extract	99.234	98.3983	95.961	92.688	84.9582
Methanolic extract	99.8607	98.468	95.8217	92.688	88.649
Hydroalcoholic extract	99.0947	98.6769	97.0056	95.5432	92.2006

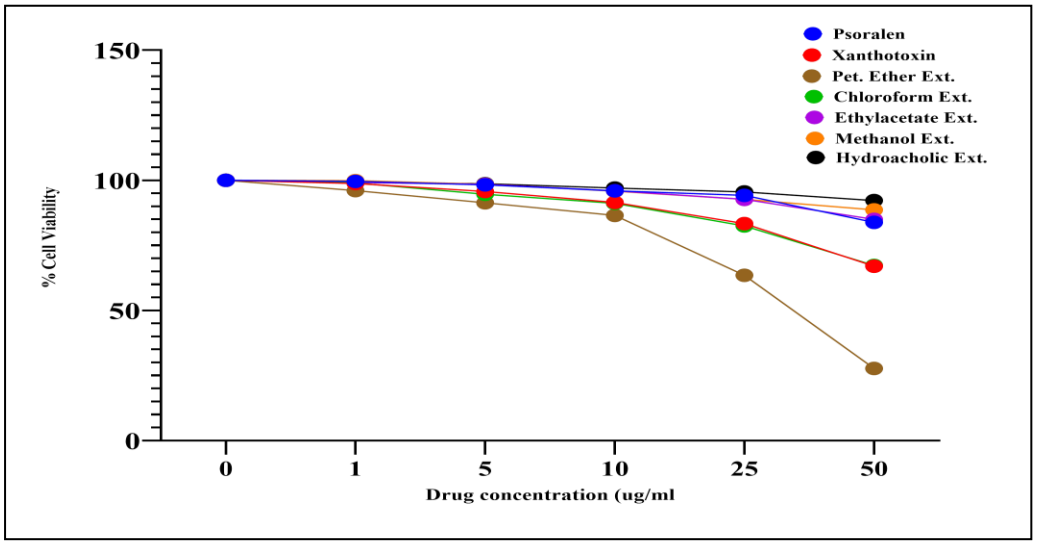


Figure 30: Overlaid graph showing the % cell viability values

The results of the study shows that the petroleum ether extract of the plant decreases the cell viability of B16F10 cells after 24 hrs of incubation with IC_{50} value of 34.49 µg/ml on B16F10 cell line. The statistical data gleaned from the MTT cytotoxicity studies leads us to infer that against B16F10 cell lines, samples namely psoralen, xanthotoxin, chloroform extract, ethyl acetate extract, methanolic extract, hydro alcoholic extract, showing moderate cytotoxic potential properties with the more than 75% cell viability at the 50 µg/ml respectively. Petroleum ether extract showed effective cytotoxicity on B16F10 cells IC_{50}.

6.2.3 Results for tyrosinase activity

Tyrosinase is a copper-containing enzyme found widely in nature. It is a rate-limiting enzyme that catalyzes the two initial sequential oxidations of L-tyrosine in melanin biosynthesis. During melanogenesis, tyrosinase interacts primarily with L-tyrosine as its substrate and catalyzes the hydroxylation of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-DOPA) and the oxidation of L-DOPA to generate DOPA quinine.

Table 38: Comparative % Tyrosinase activity observed in B16F10 cells – 48 hrs

Test group	Concentration (µg/ml)			
	1	5	10	25
Psoralen	109.408	118.803	133.592	156.268
Xanthotoxin	104.225	112.676	124.648	147.183
Petroleum ether Ext.	101.056	107.93	112.324	127.113
Chloroform Ext.	105.704	108.634	126.197	138.746
Ethyl acetate Ext.	105.634	113.732	120.07	127.465
Methanolic Ext.	101.408	107.662	110.648	115.577
Hydroalcoholic Ext.	102.099	106.366	108.408	109.394

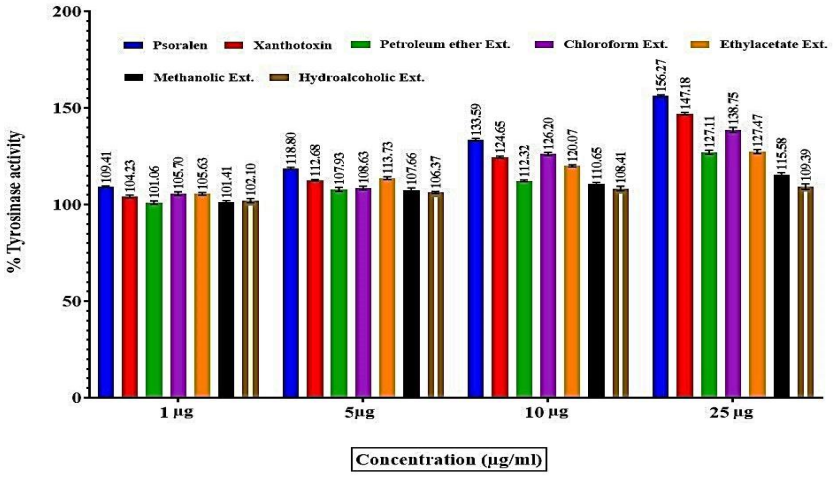


Figure 31: Comparative % Tyrosinase activity observed in B16F10 cells.

The interpretation in statistical data of tyrosinase activity study indicates a dose-dependent effect on Tyrosinase activity for both psoralen and xanthotoxin and some plant extracts. Psoralen exhibits a steady increase in tyrosinase levels with increasing concentrations, reaching a maximum of 156.27% at 25 µg/ml. Similarly, xanthotoxin shows a concentration-dependent increase, with a maximum activity of 147.18% at 25

μg/ml. Among the plant extracts, the chloroform extract demonstrates notable increase in tyrosinase, reaching 138.74% at 25 μg/ml. In contrast, the hydroalcoholic extract shows a less pronounced effect. These findings suggest a potential role for chloroform and ethyl acetate extracts of the plant as inhibitors of tyrosinase activity in B16F10 cells, highlighting the importance of concentration-dependent responses.

6.2.4 Melanin estimation study

Melanin gives skin colour and absorbs UV radiation to protect it. Melanin absorbs UV light and converts them into safe heat through 'ultrafast internal conversion' due to its photochemical properties. However, excessive melanin can cause hyper pigmentation, melasma, freckles, age spots, and senile lentigo. Thus, controlling melanogenesis is essential for treating cosmetic and serious skin diseases caused by uneven skin pigmentation. The comparative percentage melanin content values secreted in B16F10 cells– 48 hrs of the test Compounds, psoralen, xanthotoxin, chloroform extract, ethyl acetate extract, methanolic extract, hydro alcoholic ext., petroleum ether extract in given below in the table 39.

Table 39: Comparative percentage melanin content values secreted in B16F10 cells

Test groups	**Concentration (μg/ml)**			
	1	**5**	**10**	**25**
Psoralen	110.063	124.686	146.226	184.906
Xanthotoxin	104.56	112.264	134.119	156.604
Petroleum ether extract	102.516	110.377	129.874	146.226
Chloroform extract	105.189	123.428	138.679	155.031
Ethyl acetate extract	101.415	111.95	126.887	143.239
Methanolic extract	102.673	117.767	137.264	150
Hydroalcoholic extract	101.572	104.874	112.579	116.981

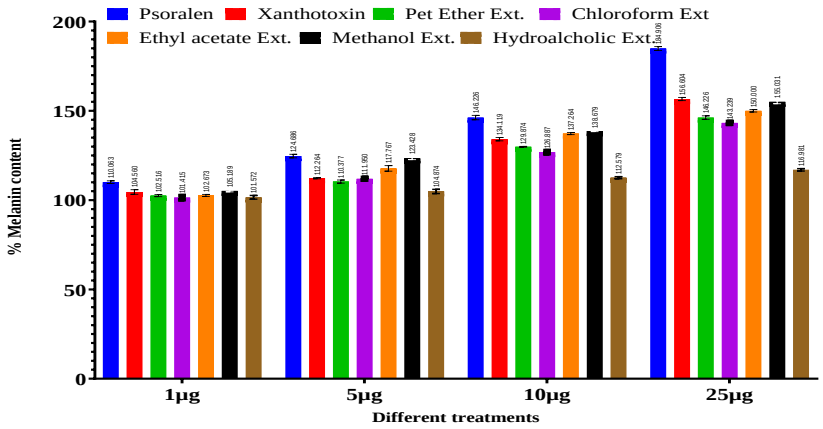


Figure 32: Comparative % melanin content secreted in B16F10 cells– 48 hrs

The data reveals concentration-dependent effects on melanin secretion by B16F10 cells in response to psoralen, xanthotoxin, and various plant extracts. Psoralen exhibits a dose-dependent increase in melanin content, reaching 184.90% at 25 µg/ml thus, indicating potential stimulatory effects on melanogenesis. Xanthotoxin demonstrates a similar concentration-dependent pattern, with a maximum melanin content of 156.60% at 25 µg/ml. Also notably; xanthotoxin appears to induce melanin synthesis to a smaller amount compared to psoralen. Among the plant extracts, the chloroform extract induces a significant increase in melanin content, reaching 155.03% at 25 µg/ml. This suggests a potential role in modulating melanin production. In contrast, the hydro alcoholic and petroleum ether extracts show comparatively lower effects.

6.3 Conclusion

Furanocoumarins constitute a class of organic compounds, encompassing both naturally occurring and synthetic derivatives. These compounds find application in the field of phototherapy for the treatment of specific dermatological conditions, certain lymphomas, and autoimmune disorders (Gasparro, 1994; Parrish *et al*., 1982). Furocoumarins exhibit pharmacological properties that remain efficacious even in the absence of irradiation. These compounds demonstrate notable activity in addressing psychological depression and display potential as therapeutic agents for the management of multiple sclerosis. This

potential is attributed to their capacity to effectively inhibit potassium channels (Wulff *et al*., 1998).

Furanocoumarins, bioactive natural compounds, are predominantly present in Umbelliferae and Rutaceae plant families, exemplified by celery (*Apium graveolens*), carrots (*Daucus carota*), and parsnips (*Pastinaca sativa*). Coumarins possess a wide array of therapeutical characteristics, including cutaneous photosensitizing, estrogenic, anti-bacterial, hypnotic, anti-helmintic, sedative, vasodilatory, analgesic actions (O'Kennedy & Thornes, 1997). Furanocoumarins possessing a tricyclic structure resembling that of psoralen exhibit notable activity as potent photosensitizers (Brown, 2001; Lozhkin & Sakanyan, 2006). The process underlying the repigmentation of vitiligo lesions through psoralen therapy remains insufficiently elucidated, given the current comprehension of the etiology of leucoderma. Nevertheless, it is reasonable to propose that the repigmentation of leucoderma may be attributed to one or more of the subsequent mechanisms (Couperus & Angeles, 1954; Swift & Angeles, 1960). Pang *et al*. (2017) synthesized coumarin derivatives with oxazole moieties from appropriate bromomethyl-isoxazoles and methylumbelliferone as melanogenic stimulators. These derived compounds outperformed 8-MOP in terms of formation of melanin in B16murine cell line (Pang *et al*., 2017).

In early 2005, the impact of seven ethanolic extracts from Umbelliferae crude medicines and sixteen extracted coumarins affecting melanin content in murine B16 melanoma cells was investigated (Matsuda *et al*., 2005b). The *H. lanatum* extract, together with the compounds psoralen, isopimpinellin, xanthotoxin, bergapten, and sphondin, exhibited a potent stimulative impact on the pigmentation. Chronic skin depigmentation can be caused by substances such as monobenzyl ether of hydroquinone (Harris, 2017); there have also been isolated reports of people acquiring vitiligo as a result of viral infection (Kumar *et al*., 2016; Philips *et al*., 2012; Pichler *et al*., 2005; Seçkin *et al*., 2004). To this day, treating vitiligo is one of the most difficult dermatological issues. Recognizing that vitiligo is more than just a cosmetic disorder and that there are effective and dependable treatments is an important first step in treating the

illness (Ezzedine *et al*., 2015). The evolving vitiligo treatment has a varied response rate. Rotational treatment is frequently required to reduce adverse effects and produce improved repigmentation response (Halder & Chappell, 2009). Furthermore, because each organism is unique, each person responds differently and unpredictable to numerous therapies (Tatu & Nwabudike, 2017a, 2017b).

Over the past decades, researchers have analyzed and recognized numerous of plant preparations as potential agents for whitening human complexion (Zhu & Gao, 2008). Nevertheless, there is a scarcity of publications demonstrating the hyper pigmentation activity of herbal extracts. The Heracleum genus is widely used to cure patches and vitiligo; however no research has been conducted to prove its efficacy. By performing studies through various *in vitro* models, we have shown that the ethyl acetate and chloroform extracts obtained from the aerial parts of *H. candicans* showed promising anti-vitiligo properties. In addition to this, anti-oxidant studies of the various extracts of the *H. candicans* suggest that the plant has good anti-oxidant potential as well. Similar anti-oxidant properties have been reported in other plants of the family like *H. persicum* (Dehghan *et al*., 2017).

In our study, we found that the ethyl acetate extract and chloroform extract of the plant show good *in vitro* anti-vitiligo and anti-oxidant potential than that of the other extracts of the plant. These may be attributed to occurrence of furanocoumarins in *H. candicans* plant. Same has been confirmed by the identification of various marker compounds by various researchers. In our study, we identified two main marker compounds i.e. psoralen and xanthotoxin by using HPLC and HPTLC techniques. These two compounds are known to possess anti-vitiligo properties and same has been confirmed by the results of our study. Based on the literature reports up to this date, this perhaps is the first such report on the anti-vitiligo activities of the *H. candicans* plant which can be very helpful in the treatment of this disease. By employing various *in vitro* models, we have shown that the traditional claim of this plant being used for anti-vitiligo (Joshi & Tyagi, 2011) has been reconfirmed by our results of the study.

Based on the outcomes of *in vitro* activities for anti-vitiligo, ethyl acetate extract and the assessment will involve the evaluation of the *in vivo* activities of chloroform and ethyl acetate extract in an ointment form. Moreover, on the basis of phytochemical characterization, the plant *Heracleum candicans* is shown to possess various furanocoumarins i.e. psoralens, xanthotoxin, etc. which have been used to treat vitiligo, leprosy, and psoriasis since ancient times (Fitzpatrick & Pathak, 1959). Esmat *et al*. (2017) and Xiao *et al*. (2015) found that furanocoumarins, originally found in *Psoralea corylifolia*, improves vitiligo repigmentation when used in conjunction with sun exposure. Despite the fact that psoralens are the most routinely utilized medications in clinical settings, the unclear targets of these chemicals have hampered vitiligo mechanism investigation and therapeutic development. Concerning the mechanism of action of psoralen derivatives, it has been proposed that they may boost melanin production by increasing the activity of tyrosinase, which has also been validated by further investigations (Sun & XU, 1994; Wu *et al*., 2000).

CHAPTER 7 DEVELOPMENT, EVALUATION AND CHARACTERIZATION OF OINTMENT

Herbal formulations refer to specific dosage forms containing one or more herbs or processed herbs in specified quantities, with the intent of delivering nutritional, cosmetic, or therapeutic benefits for modifying the physiological processes or framework of humans or animals, as well as for the evaluation, therapy, or management of medical conditions affecting humans or animals. These formulations may utilize commonly employed dosage forms in the fields of food, cosmetics, or pharmaceuticals. They can also draw from traditional medicine's known dosage forms, suitable for both external and internal administration. During the formulation development process, it is crucial to ensure the uniform distribution of processed herbs and their stability within the dosage form (Patwardhan, 2007). The potential for synergistic applications of herbal formulations exists in both developed and developing countries. Consequently, it is imperative to design these formulations effectively and evaluate their pharmaceutical properties to harness their full potential. Herbal preparations often incorporate plant materials in either finely sieved or extracted forms into a base (Al-Achi, 2008). However, these preparations are not suitable for deep wounds (Bascom, 2002). In comparison to other liquid dosage forms, herbal formulations are generally stable (Aulton, 2007). Yet, the inclusion of phytochemicals in herbal ointments can lead to product degradation. Preserving the potency of natural remedies is crucial for accurate labeling guidelines regarding storage and shelf-life (Zdoryk *et al*., 2014).

7.1 Ointments

Herbal drugs are often prepared as ointments, which are semisolid topical applications for various body surfaces, including the mucous membranes of the skin and eyes, nose, and

vagina, etc. Ointments can be medicated, containing dissolved, suspended, or emulsified medicaments in the base, serving multiple functions like protection, antiseptic action, emollient, antipruritic, keratolytic, and astringent properties. Ointment bases are typically anhydrous and can be oleaginous, absorption, water-removable, or water-soluble, with further classification into epidermatic, endodermatic, or diadermatic based on their action level. There are five ointment base types based on physical composition (Carter, 1987). In accordance with their physical makeup, ointment bases can be divided into five distinct groups. Each possesses distinctive features and differs in how well it works as a treatment (Gennaro, A.R. 18th edition) i.e.,

- Oleaginous bases
- Water in oil emulsifying bases
- Oil in water emulsifying bases
- Absorption bases
- Water soluble or water miscible bases

The base for the ointment is made, and then the active components are added to it via the trituration procedure to get the optimal ratio for the formulation. Medicated ointments are used topically for a variety of functions, including protection, disinfectant, moisturizer, antipruritic, keratolytic, and astringent properties. The medications are dissolved, suspended, or emulsified in the base (Michael, 2007). The quality of the ointment must be smooth and without any grittiness (Bhagurkar *et al*., 2016). Ointments are suitable dosage forms for drugs with a bitter taste. Ointment bases are typically hydrophobic and often comprise one or more medications in a dispersion, suspension, or solution (Kotiyal *et al*., 2020; Pawar & Gaud, 2009). Wool fat has an emollient quality and isn't easily taken; nevertheless, when mixed with appropriate vegetable oil or softened paraffin, it yields a cream that can penetrate the skin and assist in absorbing chemicals that are therapeutically useful. A typical ointment's emollient qualities are improved by

Cetostearyl alcohol, while hard paraffin acts as a hardening agent. Soft paraffin, either white or yellow, functions as both an emollient and protective ointment basis for topical activity (Jain & Gupta, 2008).

7.2 Experimental

7.2.1 Preparation of an ointment base using fusion method

This technique involves combining the constituents of base in glass beaker and melting them combined at 70°C. Once melted, the constituents are gently agitated while upholding the temperature at 70°C, over a specific duration. Subsequently, the mixture is cooled with continuous stirring. Using a mortar and pestle, the active components are ground into the base to formulate an ointment mixture. After that, the remaining ointment was filled in tubes and maintained at ambient temperature.

7.2.2 Preparation of an ointment by using trituration method

Three anhydrous topical ointment bases were created using the fusion method. This involved placing the base constituents in a pan and allowing them to melt at 70°C. After melting, the constituents were gently agitated by maintaining the ambient temperature for about 5 mins, afterwards constantly mixing while it cooled (Rajalakshmi *et al*., 2010). The ointment formulation was achieved by incorporating 1 g of dried chloroform extract of *H. candicans* into the various ointment bases through trituration in a mortar (ceramic) using a pestle. During this study, an ointment of chloroform and ethyl acetate extracts was prepared in an ointment base at a concentration of 10% w/w, consistent with a previous study on the fabrication of an anti-vitiligo ointment from *Psoralea corylifolia*, which also used a 10% concentration (Hussain *et al*., 2016). Subsequently, after being prepared, the medicinal ointments were put into the containers, labeled, and kept at the ambient temperature until they underwent testing.

7.2.3 Composition of the formulations on the basis of ointment base used

Table 40: Composition of formulations of (F1, F2, F3) chloroform extract

F1	Quantity	F2	Quantity	F3	Quantity
Chloroform extract	1.0 g	Chloroform extract	1.0 g	Chloroform extract	1.0 g
Liquid paraffin	2.0 g	Hard paraffin	5.0 g	Liquid paraffin	2.2 g
White soft paraffin	5.0 g	Cetostearyl alcohol	5.0 g	Cetostearyl alcohol	2.8 g
Emulsifying wax	3.0 g	Wool fat	5.0 g	White soft paraffin	5.0 g
-	-	White paraffin	8.5 g	-	-

Table 40 outlines three different formulations (F1, F2, F3) along with the quantities of ingredients used in each formulation. The quantities of ingredients are carefully measured and combined to achieve the desired properties and characteristics of the final product. Additionally, the table provides a clear and structured overview of the formulations and their ingredient compositions, aiding in replication, quality control, and further analysis.

Formulation F1: This formulation includes chloroform extract, liquid paraffin, white soft paraffin, and emulsifying wax. Liquid paraffin, white soft paraffin, and emulsifying wax are commonly used in pharmaceuticals and cosmetics for their emollient, lubricating, and emulsifying properties (Abhishek & Krishanu, 2021), I.P, 1966.

Formulation F2: This formulation contains chloroform extract, hard paraffin, cetostearyl alcohol, wool fat, and white paraffin. Hard paraffin and white paraffin likely contribute to the consistency and texture of the final product. Cetostearyl alcohol is a mixture of cetyl

and stearyl alcohols commonly used as an emollient and emulsifier in skincare products. Wool fat (lanolin) is a natural wax secreted by the sebaceous glands of sheep and is often used as a moisturizer and emollient (Sawant & Tajane, 2016).

Formulation F3: This formulation comprises chloroform extract, liquid paraffin, cetostearyl alcohol, and white soft paraffin. Similar to the other formulations, these ingredients likely serve various purposes such as emollient, emulsifying, and lubricating properties (Abhishek & Krishanu, 2021). Overall, a systematic breakdown of ingredients and quantities for different formulations, aiding in the preparation of ointment from the plant extracts is given below in the table 41-42 as;

The table 41 presents three formulations (F1, F2, F3) alongside the quantities of ingredients used in each. These precise measurements are crucial for achieving the desired properties of the final product. The structured layout aids in easy replication, quality control, and further analysis of the formulations. The concentration of herb extract used was 10% w/w (25-times of EC_{50}) according to anti-oxidant activity of herb extract (EC_{50} = 3.805 mg/ml) (Gani *et al*., 2020).

Table 41: Composition of different formulations of ethyl acetate extract

F1	Quantity	F2	Quantity	F3	Quantity
Ethyl acetate extract	1.0 g	Ethyl acetate extract	1.0 g	Ethyl acetate extract	1.0 g
Liquid paraffin	2.0 g	Hard paraffin	5.0 g	Liquid paraffin	2.2 g
White soft paraffin	5.0 g	Cetostearyl alcohol	5.0 g	Cetostearyl alcohol	2.8 g
Emulsifying wax	3.0 g	Wool fat	5.0 g	White soft paraffin	5.0 g
-	-	White paraffin	8.5 g	-	-

7.2.4 Evaluation and characterization parameters for ointment

7.2.4.1 Color and odor: It was determined by visual examination.

7.2.4.2 Homogeneity: Visual inspection was used to verify the uniformity of each created ointment. They underwent an examination to make sure there were no lumps on them.

7.2.4.3 Loss on drying: The ointment was placed in a Petri dish and then placed on a water bath and dried at 105°C to measure the loss on drying.

7.2.4.4 pH: A pH meter with a digital sensor was employed to measure the pH of different compositions. After being dissolved in 100 ml of purified water, 1 g of ointment was kept for a period of two hrs. Three separate measurements for every formulation's pH were made.

7.2.4.5 Topical sensitivity test: Ointments were assessed for sensitivity to the skin by putting them to a sample of human respondents' elbows and watching for any negative effects, including skin inflammation, irritation, skin reddening, etc.

7.2.4.6 Diffusion studies: To assess the diffusion of ointment through agar medium.

7.2.4.7 Spreadability: It was ascertained by gradually rubbing the ointment over the surface of the skin.

7.2.4.8 Washability: It was examined by washing the skin area of ointment application in normal water over roughly ten mins.

7.2.5 Stability studies

At multiple temperatures, stability assessments were performed for the developed formulations (4°C, 25°C and 37°C) for a period of 3 months.

7.3 Results of the study

7.3.1 Evaluation of herbal ointment from the extracts of Heracleum candicans

This study focuses on the comprehensive physicochemical characterization of three formulations (F1, F2, and F3) containing chloroform extract intended for topical

application. Parameters such as color, odour, loss on drying, non-irritancy, pH, diffusion study, spreadability, extrudability, solubility, consistency, washability, and viscosity were evaluated.

Table 42: Evaluation of Chloroform extracts ointment

In order to assess the physical and quality attributes of the developed ointment, the evaluation of the prepared ointment is carried out which involves an array of parameters like color, odour, loss on drying, non-irritancy, pH, diffusion study, spreadability, extrudability, etc. The results of the evaluation are summarized below in the table as;

S. No.	Parameters	Formulations		
		F1	F2	F3
1.	Color	Darkish brown	Greenish black	Dark green
2.	Odor	Characteristic	Characteristic	Characteristic
3.	Loss on drying (%)	34.36±1.02	24.24±1.21	25.24±1.33
4.	Topical sensitivity	Not irritating	Not irritating	Not irritating
5.	pH	6.3±0.1	6.6±0.2	6.4±0.4
6.	Diffusion studies (cm)	0.65±0.025	0.78±0.042	0.70±0.018
7.	Spreadability (sec.)	6.8±0.56	7.7±0.47	7.0±0.29
8.	Extrudability (g)	0.51±0.010	0.54±0.025	0.40±0.032
9.	Solubility	Soluble in ethyl acetate, slightly soluble with methanol, ethanol and chloroform, slightly soluble in water	Soluble in ethyl acetate, slightly soluble with methanol, ethanol and chloroform, slightly soluble in water	Soluble in ethyl acetate, slightly soluble with chloroform, methanol, ethanol and slightly soluble in water
10.	Consistency	Smooth	Smooth	Smooth
11.	Homogeneity	Good	Good	Good
12.	Washability	Good	Good	Good
13.	Viscosity (cp)	6415	6424	6433

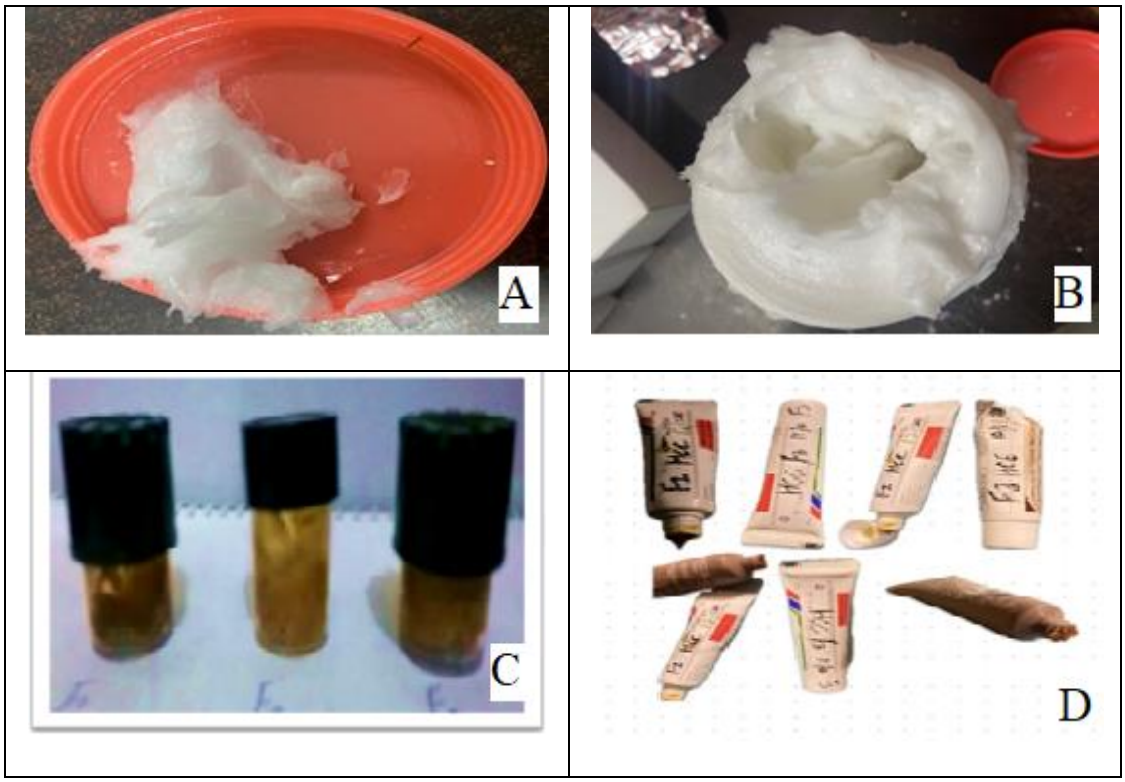


Figure 33: Formulation of ointment A-D: (A-B= prepared ointment base; C= ointment of extracts; D= Storage of ointment in tubes)

7.3.2 ***Color and Odour:*** The formulations exhibit distinct colors, with F2 showing a greenish brown hue and F3 displays a dark greenish brown color. All formulations have characteristic odors, indicating the presence of specific components.

7.3.3 ***Loss on Drying:*** F2 demonstrates a significantly lower percentage of loss on drying (24.24 ± 1.21%) compared to F1 (34.36 ± 1.02%) and F3 (25.24 ± 1.33%). This suggests differences in water content and potential implications for stability.

7.3.4 ***Non-irritancy:*** All formulations are identified as non-irritants, indicating their safety for topical use. The results are as per the observations reported by the volunteers for evaluation of the developed ointment.

7.3.5 ***pH:*** The pH values of F1, F2, and F3 fall within a close range, suggesting similar acidic to neutral pH conditions suitable for skin application.

7.3.6 Diffusion Study, Spreadability, Extrudability:

F2 demonstrates slightly higher diffusion (0.82 ± 0.016) and spreadability (7.7 ± 0.47 sec) compared to the other formulations, while F3 exhibits the lowest extrudability (0.40 ± 0.032 g). These parameters are crucial for effective topical application and absorption.

7.3.7 Solubility:

All formulations exhibit similar solubility profiles, being soluble in ethyl acetate and miscible with various solvents, suggesting potential versatility in formulation.

7.3.8 Consistency and Washability:

The formulations show a smooth consistency and good washability, contributing to user-friendliness.

7.3.9 Viscosity:

The negligible differences in viscosity (6415 cp., 6424 cp., and 6433 cp.) suggest comparable rheological properties among the formulations.

7.4 Conclusion

This detailed physicochemical characterization provides valuable insights into the properties of chloroform extract formulations. All formulations demonstrated no skin irritation, such as erythema and edema, when applied to the skin for a week. The rheological analysis using a Rotational Brookfield Viscometer revealed an inverse relationship between spindle speed and viscosity; as spindle speed increased, viscosity decreased. When viscosity and spreadability were compared, it was discovered that spreadability reduced with increasing viscosity and vice versa. Notably, the F2 formulation of the chloroform extract of the plant exhibited the most favorable results among the three formulations. A diffusion study conducted after 60 min indicated that the F2 formulation displayed superior diffusion through the medium compared to the other two formulations. F2 stands out with its lower loss on drying, higher diffusion, and spreadability. It thus exhibit promising attributes for further development and consideration in topical applications, pending additional studies on its stability.

7.5 Stability study of developed formulations of chloroform extract

Stability studies are crucial for ensuring product superiority and safety, preventing the development of adverse compounds resulting from the degradation of active ingredients. They also provide information on the shelf life of newly prepared drugs and their storage conditions (Neeraj & Sindhu, 2013). Researchers have conducted stability testing on typical natural plant extracts, particularly flavonoid-containing herbal drugs, to assess the stability of natural products (Heigl & Franz, 2003). Reports on stability testing of herbal medicinal products, along with discussions of potential resolution approaches for problematic cases, have also been documented (Poetsch *et al*., 2006). The efficacy of items employed topically is greatly influenced by a formulation's viscosity, which is strongly linked to properties including stability, drug absorption, dissemination, and simplicity of use (Kumar *et al*., 2014). Spreadability refers to the convenience by which a material that is semi-solid covers the surface of the skin (Khar, 2013), whereas the convenience by which a semi-solid can be squeezed from a container is referred to as extrudability (Khar, 2013). Better extrudability is indicated by a greater quantity of substance being dispensed (Panwar *et al*., 2011). Stability studies of herbal formulations are essential not only during production but also for assessing storage conditions and shelf life over time (Neeraj & Sindhu, 2013). Stability, defined as maintaining quality until the expiration of the indicated shelf-life (Lang, 2001), aims to ensure that a drug and its product maintain established specifications for identity, strength, purity, and quality. It involves maintaining predefined limits for all important characteristics over a specific time and under particular conditions, considering the potential impact of all constituents, both therapeutically active and inactive (Pingale *et al*., 2008). Stability can be influenced by factors such as the physical and chemical properties of substances, environmental factors (e.g., light, temperature, humidity), and microbiological changes (I. H. T. Guideline, 2003). Additionally, factors like particle size, pH, solvent properties, container characteristics, and the presence of other chemicals can affect stability, either due to contamination or deliberate mixing of various products (Pingale *et al*., 2008).

This study investigates the stability of formulations F1, F2, and F3of chloroform extract of *H. candicans* under diverse temperature settings (20°C, 25°C, and 37°C) by examining parameters such as color, odour, pH, and phase separation.

Table 43*:* Stability study of three developed formulations of chloroform extract

Temperature	Parameters	F1	F2	F3
2^oC	Color	Unchanged	Unchanged	Unchanged
	Odor	Unchanged	Unchanged	Unchanged
	pH	6.3±0.15	6.6±0.29	6.4±0.40
	Phase separation	Unchanged	Unchanged	Unchanged
25^oC	Color	Unchanged	Unchanged	Unchanged
	Odor	Unchanged	Unchanged	Unchanged
	pH	6.4±0.28	6.7±0.37	6.5±0.14
	Phase separation	Unchanged	Unchanged	Unchanged
37^oC	Color	Unchanged	Unchanged	Unchanged
	Odor	Unchanged	Unchanged	Unchanged
	pH	6.4±40	6.65±0.47	6.8±0.28
	Phase separation	Slight separation	Unchanged	Unchanged

The several physicochemical metrics that were used to assess the formulated ointment combinations are shown in Table 43 and summarized below as;

The pH of the developed ointment falls within the typical pH range of human skin (i.e. 6.5±1) (Bureau of Indian Standards, 2004).

7.5.1 Color and Odor Stability

Across all temperature conditions, formulations F1, F2, and F3 exhibit consistent color and odor, demonstrating robust stability in terms of sensory attributes.

7.5.2 pH Stability

At 2°C and 25°C, the pH values remain within a narrow range for all formulations, indicating a stable acidic to neutral pH. However, at 37°C, F1 shows a slight increase in pH (6.4 ± 0.41), while F2 and F3 maintain relatively stable pH values (6.65 ± 0.48 and 6.8 ± 0.30, respectively). This pH variation in F1 may necessitate further investigation to ensure stability under physiological conditions.

7.5.3 Phase Separation

At 37°C, F1 exhibits a slight phase separation, indicating a potential change in physical characteristics. This separation could be attributed to changes in the formulation's rheological properties or the solubility of certain components at higher temperatures. F2 and F3, however, show no phase separation, indicating better stability. The formulations F1, F2, and F3 demonstrate commendable stability in terms of color, odor, and pH under different temperature conditions. However, at 37°C, F1 shows a slight pH increase and phase separation, suggesting a need for further optimization. These findings are crucial for ensuring the formulations' stability during storage and potential application scenarios, warranting additional studies to understand the underlying causes of instability and refine the formulations for optimal performance. Based on a comprehensive evaluation of various parameters, it can be concluded that the F2 formulation of the chloroform extract of *H. candicans* displayed more favorable characteristics than formulations F1 and F3.

CHAPTER 8 EVALUATION OF IN VIVO STUDIES ON THE DEVELOPED OINTMENT

The functional similarity of monobenzone with tyrosine, the putative precursor of melanin production, explains the vitiligo paradigm that it induces. Upon exposure to tyrosinase, monobenzone transforms into an exceedingly reactive quinone moiety. These products interact with enzymes in the melanosome organelle, causing adaptations. This, in turn, changes their detection by T-cells, potentially disrupting immunity (Hariharan *et al*., 2011). Additionally, monobenzone might interact with immunological and reactive oxygen compound pathways to cause discoloration resembling vitiligo and the loss of melanin forming cells (Van Den Boorn *et al*., 2011).

Histopathological characteristics reminiscent of vitiligo and an autoimmune reaction to autologous melanin producing cells are shown in an animal model, where dermal treatment of monobenzone is used (Zhu *et al*., 2013). Monobenzone, as a clinical analogue of tyrosine promotes melanin cell strain, sets off pro-inflammatory signals, and worsens vitiligo patients' discoloration (Van Den Boorn *et al*., 2011). Four-week-old mice with hairless abdomens showed signs of fur discoloration at the place of shaving, which later extended to other areas such as the tail and ears. After the therapy, CD8+ T cells began to enter the afflicted epidermis. Notably, the same methodology in RAG-deficient mice induced fur to become less pigmented where the therapy was applied, compared to elsewhere (Zhu *et al*., 2013). These findings suggest that localized depigmentation at the application site may result from the direct toxicity of monobenzone; while an immunological reaction is necessary for loss of melanin to extend to other regions.

In a recent comprehensive analysis of the mouse vitiligo model, it was observed to recapitulate the key characteristics of human vitiligo (Xu *et al*., 2022). C57BL/6J mice's melanin production in hair shafts provides an invaluable *in vivo* model for investigating pigmentation (Ishikawa *et al*., 2007; Park *et al*., 2004). It's worth noting

that, unlike humans, melanocytes in mouse dorsal skin are exclusively localized within hair follicles (Wolnicka-Glubisz *et al*., 2013).

Considering the outcomes of the animal studies, it was observed that an ointment containing a 10% concentration of chloroform extract exhibited more effective anti-vitiligo activity than the ointment containing ethyl acetate extract. Notably, substantial repigmentation on the dorsal area in the mice skin was observed at the end of the study. These findings are corroborated by the data presented in Table 45, which indicates increased NAG (N-acetylglucosaminidase) activity in the case of the aerial chloroform extract in comparison to the aerial ethyl acetate extract of *H. candicans.*

Additionally, the groups that received ointments containing 10% chloroform and ethyl acetate plant extracts had a higher overall skin color ratio than the control groups. The vehicle had no impact affecting the hair color of the control group of mice, as revealed by the fact that their black hair maintained equivalent to that of regular, non-treated mice. On the other hand, every mouse in the negative control group had white hair, with a few showing a combination of black and white growth of hair. The hair color of the standard and various sample groups clearly showed that the discoloration hypothesis had been inhibited by the plant preparation. The group getting only monobenzone ointment was less likely to have testing subjects having a variety of colors, with white being most prevalent. On the other hand, mice given an ointment containing plant extracts displayed an increased black hair intensity surrounding the white areas of the dorsal surface. Coumarin-containing plants and their derivatives found across numerous regions of the globe (Niu & Aisa, 2017a) are traditionally used for skin repigmentation. These plants have demonstrated the ability to stimulate melanin formation, making them advantageous in the management of depigmentation disorder (Zaidi, 2017). Additionally, plants with compounds acting as tyrosinase inducers including *Moricandia arvensis*, *Daphne gnidium*, and *Vernonia anthelmintica*, have been employed to enhance skin pigmentation (Nakajima *et al*., 1998).

Recent studies have focused on elucidating specific mechanisms to induce melanin biosynthesis and functional melanocytes, aiming to develop novel therapeutic

agents for vitiligo (Kumar *et al*., 2016; Lee *et al*., 2000; Sato *et al*., 2016). In the case of the 10% plant extract ointment-treated mice, the restoration of black skin color suggests that *H. candicans* extract may enhance pigmentation by inducing tyrosinase enzyme activity. According to an investigation, histopathological evaluations of the perilesional edges surrounding the depigmented epidermis have demonstrated an invasion of activated T-cells alongside additional lymphocytes (Badri *et al*., 1993). Moreover, some polyphenolic compounds with antioxidative properties have shown inhibitory effects on melanogenesis in B16 cells (Wang *et al*., 2011). These findings underscore the intricate pathways involved in melanin synthesis and its potential modulation as a therapeutic approach.

8.1 *In vivo* anti-vitiligo activity in C57 mice

To extend our understanding of the therapeutic potential, an *in vivo* anti-vitiligo study was conducted on C57 mice, evaluating the effects of various extracts incorporated in the ointment. This investigation evaluated the systemic response and potential therapeutic benefits of the extracts in C57 mice, providing valuable insights into their efficacy beyond cellular studies. The utilization of C57 mice as an *in vivo* model allows for a more comprehensive assessment of the anti-vitiligo activity, considering the complex interactions within a biological system. The *in vivo* activity was duly approved by the IAEC (CPCSEA) of University of Kashmir vide approval no. IAEC-2023-01-02.

In the context of current study, it was observed that monobenzone-induced discoloration in C57 mice led to the loss of functional melanin cells and the infiltration of T-cells in the perilesional area, resembling the abrasions found in human vitiligo. These results support the notion that chloroform and ethyl acetate extracts may mitigate the severity of vitiligo by means of triggering CD8+ T-cell death and preventing CD8+ T-cell proliferation. This implies that the chloroform and ethyl acetate extracts from the *H. candicans* plant have the potential to be candidates for intervention in depigmentation. Moreover, numerous studies over the years have emphasized the connection between depigmentation and inflammation. It has been shown that a low-intensity pro-inflammatory mononuclear cell intrusion exists among the epidermis-dermis contact and

the upper layer of the dermis (Taïeb & Picardo, 2009). These findings highlight the complex interplay between immune responses, melanocytes, and skin pigmentation in vitiligo.

8.2 Experimental

8.2.1 Materials and method

8.2.1.1 Description of animals used

Species	Mice
Strain	C57
Sex	Female
Weight range at the start of experiment	22 to 25 g
Number of animals:	36
Number of groups:	4
Number of animals per group Test sample:	12 (Female)
Positive control:	06 (Female) and Negative
Control:	06 (Female)
Vitiligo inducing drug:	Monobenzone (Cat No: PHR3208, Sigma).

On allotment of mice, the experimental animals were housed individually in each PVC cage with stainless steel lids in a group wise manner in the experimental room. The rooms where animals were housed, was kept under standard conditions: temperature at 22± 3°C and relative humidity ranging between 50% and 70%. 12 hrs photo-periodicity for the light and dark was followed during the initial acclimatization and therapeutic period. The animals were given pelleted feed stuff containing standard composition of all macro and micronutrients. Reverse osmosis water collected through aqua guard was provided to animals' ad libitum.

8.2.2 Identification of animals

Animals were identified by unique cage number, study number, animal numbers, dose, group, and route of administration, species, sex and experiment starting date, Individual animals were uniquely labeled with lasting identification numbers using an indelible marker pen, a process initiated before the commencement of test item administration and renewed on a weekly basis. This methodical numbering system guaranteed precise monitoring and management of each animal throughout the study, enhancing accuracy and reliability.

8.2.3 Preparation of animals

The animals were acclimatized to experimental room conditions for duration of six days before starting the treatment and all the animals were assessed with once a day for monitoring experimental signs and twice a day for assessing mortality. The study utilized mice that were in good health conditions. Animals are randomized and assigned to the treatment groups. The fur covering the selected skin spots was individually trimmed and removed on the dosing day, just before the administration of the dose, handled with care to avoid abrading the skin.

8.2.4 Acute toxicity studies of extracts

This activity was accomplished as per Organization for Economic Cooperation and Development (OECD) guidelines no. 425. C57 mice were employed during the research. Initially one animal was administered Chloroform extract of *Heracleum candicans* with a dose of 2000 mg/kg b.w, p.o and observed over 24 hrs. The animal survived and therefore four more animals were dosed at 2000 mg/kg b.w, p.o and were observed for 24 hrs. All five animals survived. Same method was used for ethyl acetate extract of *Heracleum candicans*, using 5 animals per extract. All the animals survived indicating LD50 is higher than 2000 mg/kg body weight for all plant extracts. Based on acute studies, $1/6^{th}$ of the dose i.e., 300 mg/kg body weight was thus designated for further experimental evaluation. In accordance with the guidelines, the dosage is classified as toxic if mortality is observed in 2 or 3 animals. In the case of mortality in one animal, the

same dose is reiterated to verify its toxicity. Absence of mortality indicates the plant extracts are deemed non-toxic (OECD, 2022).

8.2.5 Experimental Procedure

It involves the estimation of vitiligo healing activity of the test compounds (plant extracts/ointments) labeled as Chloroform and Ethyl extract ointment with 10% concentration in C57 mice under *in vivo* settings. Vitiligo was induced in healthy C57 mice by using Monobenzone-40% cream. The cream was applied on test area. For vitiligo induction the dorsal skin of the C57 mice had been shaved prior to start of the dose. The Monobenzone-40% cream was applied topically on test area once a day for 50 days. Similarly, chloroform and ethyl acetate ointments were also applied in a different spot on the dorsal skin of the mice towards necks for a period of 50 days (Zhu *et al*., 2013).

8.2.6 Estimation of N-acetyl-b-D-Glucosaminidase (NAG) activity

Rauh *et al*. (2011) utilized a technique to assess enzymatic function. In brief, 6 mm circular tissue samples were homogenized for 45 seconds at 0°C using a motor-driven homogenizer in a solution consisting of 0.75 ml 80 mM PBS (pH 5.4) and 0.5% HTAB. After centrifugation at 11,200 rpm for 20 min at 4°C, 25 µl of the supernatant was applied to 96-well plates in triplicates. Subsequently, 100 µl of 50 mM citrate buffer (pH 4.5) and 25 µl of 2.24 mM NAG were added, followed by incubation at 37°C for 1 hr. The reaction was concluded with 30 µl of glycine buffer (200 nM, pH 10.4). Enzymatic activity, assessed at 405 nm using a plate reader, quantified tissue samples' optical concentration in terms of mOD (Rauh *et al*., 2011).

8.3 Results

Before the start of the experiment, the animals were given test dose of the ointment on the selected spots so as to check for any skin allergies. The animals were observed for 3 hrs to check for any signs of skin rashes, redness, and edema. After this, the test drugs

were locally applied in an ointment form at a 10% concentration for duration of 50 days. Following this treatment period, the vitiligo that had developed on the skin of C57 mice gradually began to disappear. On the 14th day of the test, in the positive control group (comprising 40% monobenzone-treated mice), small white patches first appeared, which subsequently expanded. In contrast, fewer white patches were observed in the test groups. By the 21st day after monobenzone treatment, distinct regions of depigmentation (white patches on skin areas not exposed to test formulations) became visible over the ears, trunk, of some mice. The prevalence of these patches in the standard control group was notably high at 64.41%, while in the test groups, it was considerably lower at 35.59%. The difference between standard group and chloroform and ethyl acetate extracts treated was statistically significant. Also, the reduction in depigmentation observed with the application of chloroform and ethyl acetate extracts suggests that these test samples not only decrease the occurrence but also impede the growth of vitiligo in the test animals. Among the provided extracts, the ointment containing chloroform extract exhibited a more potent anti-vitiligo effect than the one with ethyl acetate extract in the 40% monobenzone-induced vitiligo model.

8.3.1 Histological analysis of animal skin tissues after 50 days post treatment

Following a 50-day post-treatment period, we conducted histological analysis on treated animal skin tissues. This assessment aimed to unveil microscopic details, revealing structural changes, cellular responses, and tissue regeneration linked to the treatment. The extended timeframe facilitated a thorough evaluation of long-term effects, providing insights into the enduring impact on the skin's histological architecture.

8.3.1.1 Histological analysis of various test groups

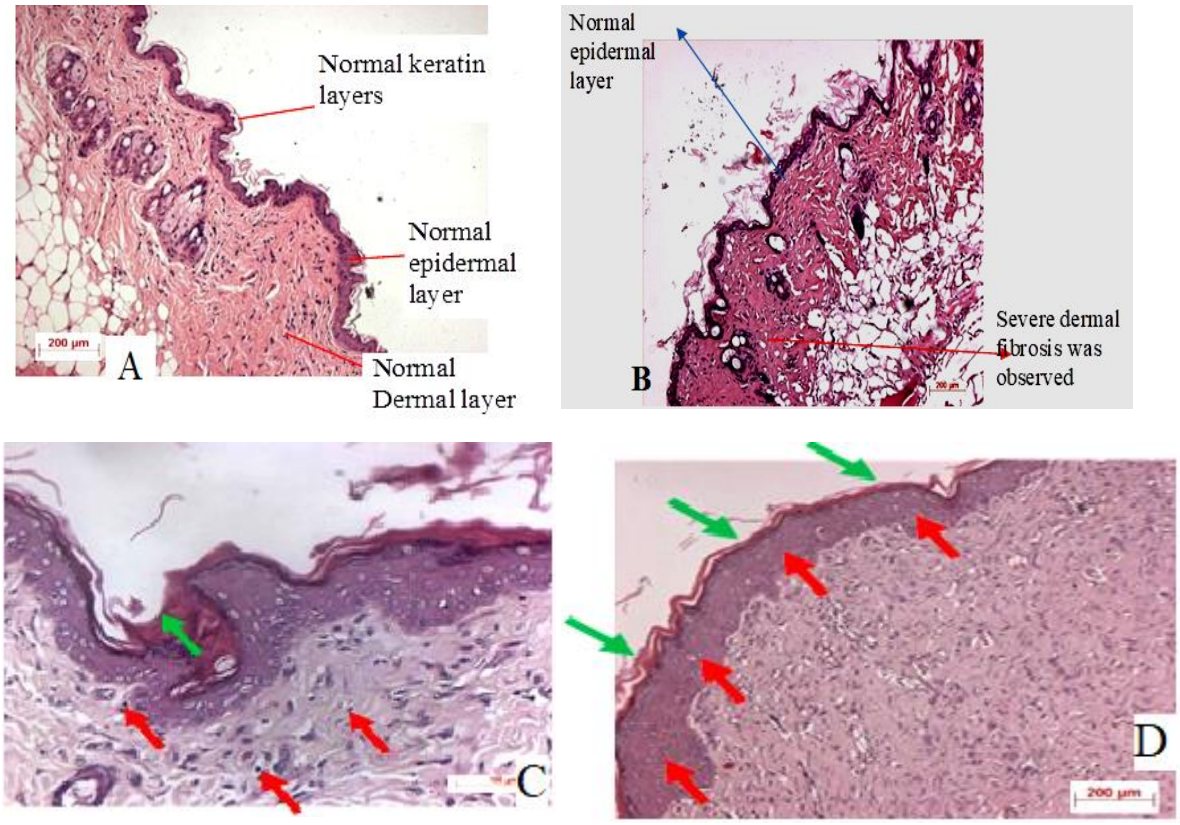


Figure 34: Histological analysis of skin tissues of C57 Mice A-D: (A= Negative Control (Distilled water); B= Positive Control (40% Monobenzone); C-D = Chloroform extract (10%) ointment)

The histological evaluation of the chloroform treated group revealed- acanthosis, Moderate epidermal hyperplasia specifically hyperplasia of stratum spinosum layer – red arrow; Mild hyperkeratosis of epidermal layer of skin green arrow; Moderate dermal fibrosis with proliferation of fibrous tissue – red arrow.

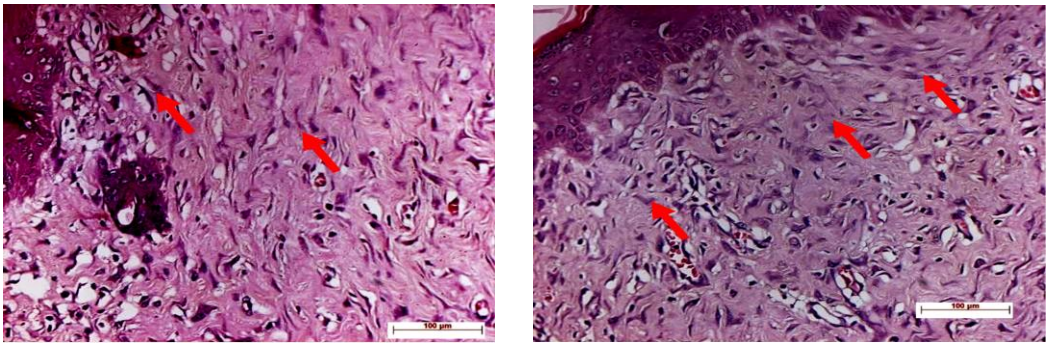


Figure 35: Histological analyses of chloroform extract (10%) ointment- showing infiltration of few inflammatory cells, milk hyperkeratosis of epidermal layer of skin.

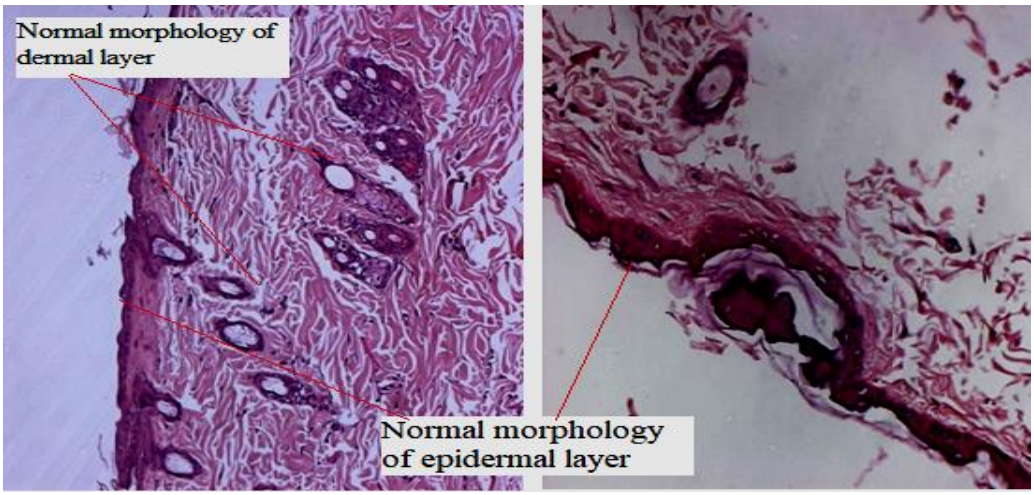


Figure 36: Histological analyses of chloroform extract (10%) ointment- showing infiltration of few inflammatory cells, milk hyperkeratosis of epidermal layer of skin.

8.3.2 Individual animal body weights

The individual body weights of all the test animals were noted on Day 1^{st,} Day 12th, Day 24th, Day 36th and Day 48th for any variations. The results of the study did not show any major variations in individual weights of animals after 48 days of treatments as represented in table 44.

Table 44: Individual animal body weights after dose (mg/kg body weight)

Group/Dose	Animal No.	Body weight (g)				
		Day 1	Day 12	Day 24	Day 36	Day 48
G1 (Chloroform extract)	1	23.5	24.2	25.0	25.7	26.4
	2	22.6	23.1	23.7	24.5	25.1
	3	22.7	23.4	24.1	24.6	25.2
	4	22.9	23.4	24.3	24.9	25.4
	5	23.5	24.2	24.8	25.5	26.2
	6	24.5	25.4	26.7	27.1	27.8
G2 (Ethyl acetate extract)	7	24.6	25.1	25.7	26.4	27.1
	8	25.2	25.8	26.4	27.2	27.9
	9	22.3	23.4	24.5	24.8	25.6
	10	22.6	23.4	23.8	24.8	25.7
	11	22.5	23.1	24.2	24.8	25.2
	12	22.6	23.4	23.9	24.5	24.8
G3 (Negative control)	13	23.7	24.2	25.6	26.3	26.7
	14	22.8	23.3	24.8	25.6	26.2
	15	23.5	24.2	24.8	25.5	26.8
	16	23.3	23.8	24.4	25.2	26.4
	17	23.8	24.5	25.6	26.3	27.2
	18	24.2	25.4	26.2	26.8	27.6
G4 (Positive control)	19	24.6	25.2	26.4	26.8	27.4
	20	22.3	23.1	23.5	24.4	25.0
	21	23.0	23.8	24.2	24.5	25.3
	22	24.7	25.5	25.9	26.3	27.1
	23	24.1	24.6	24.9	25.4	25.9
	24	22.5	23.1	23.9	24.5	25.0

8.3.3 Results of NAG concentration in different conditions of vitiligo

This analysis aims to elucidate the dynamic changes in NAG levels associated with different stages or treatments of vitiligo, providing valuable information about the enzymatic activity and its potential implications in the pathophysiology of the

disorder.The data indicate that both the chloroform and ethyl acetate extracts, when formulated as 10% ointments, may have potential anti-vitiligo effects, as evidenced by the reduced NAG concentrations compared to the Monobenzone-treated group as shown in table 45. Further investigations, including histological analyses and assessments of other inflammatory markers, are warranted to elucidate the mechanisms underlying the observed effects and to gauge the translational potential of the test plant extracts of *H. candicans* in mitigating vitiligo.

Table 45: NAG concentration in different conditions of vitiligo (N=6±SD)

Condition	Concentration	NAG conc. (U/L) ± SD
G1-Control	Distilled water	1.66 ± 0.41^{d}
G2-Diseased (Monobenzone)	40% ointment	93.9 ± 4.05^{c}
G3-Chloroform extract	10% ointment	15.89 ± 1.97^{a}
G4-Ethyl acetate extract	10% ointment	24.94 ± 1.45^{b}

Where a, b, c, d indicate the significance of values reported during the evaluation on the basis of various P values. The mean values in a given row that are not accompanied by the same letter are deemed significantly different (P value ≤ 0.05). The values indicate the mean ± standard deviation (n = 3).

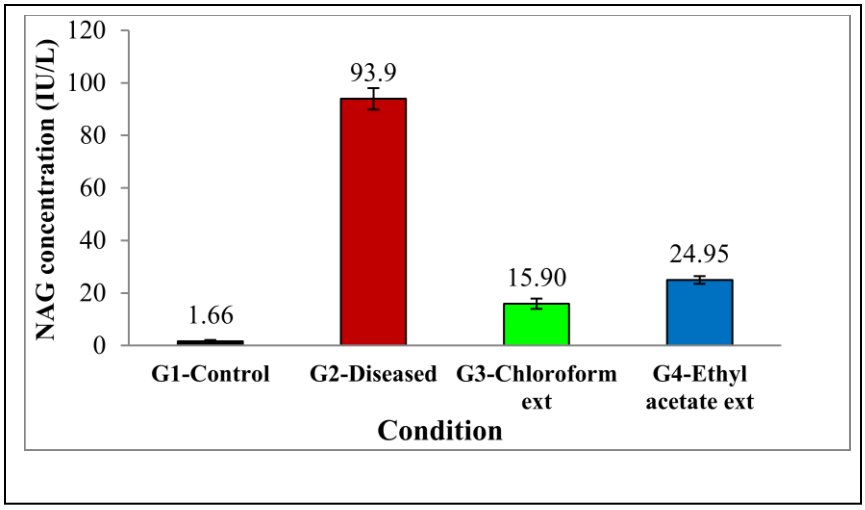


Figure 37: NAG concentration in different conditions of Vitiligo

Control Group (G1): The low NAG concentration in the control group treated with distilled water (1.66 ±0.41 U/L) suggests the baseline level of NAG in normal skin conditions.

Diseased Group (G2): The significant elevation in NAG concentration in the diseased group treated with Monobenzone (93.9 ±4.05 U/L) is noteworthy. Monobenzone is known to induce depigmentation and has been linked with inflammatory responses and oxidative stress. The substantial increase in NAG levels may indicate tissue damage and inflammation.

Chloroform Extract Group (G3): The group treated with chloroform extract shows a markedly lower NAG concentration (15.89 ±1.97 U/L) compared to the diseased group. This reduction could imply a potential mitigating effect of the chloroform extract on inflammation or tissue damage associated with Monobenzone treatment.

Ethyl Acetate Extract Group (G4): Similar to the chloroform extract group, the ethyl acetate extract group exhibits a lower NAG concentration (24.94 ±1.45 U/L) compared to the diseased group. This suggests that the ethyl acetate extract may also have a protective effect against the inflammatory response induced by Monobenzone.

8.3.4 Skin and Hair Depigmentation Assessment

In the various treatment groups, observers objectively quantified the extent of depigmentation. Some of the photographs of the animal test groups are given below as;

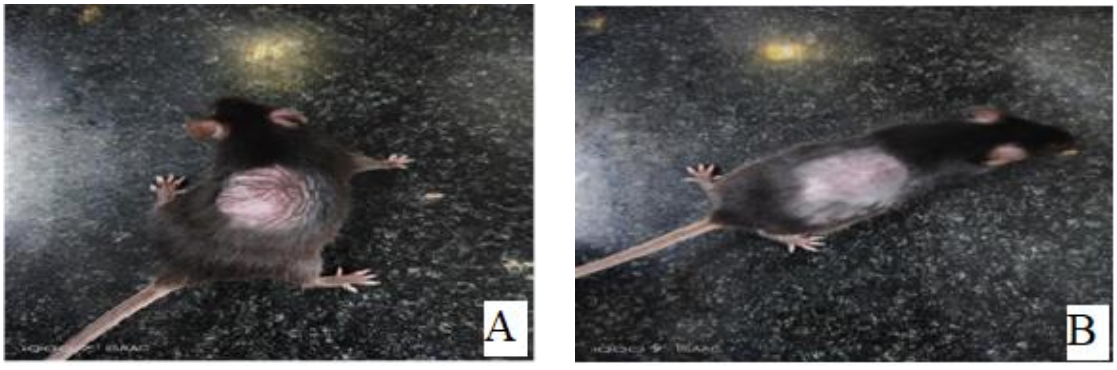


Figure 38: Photographs of C57 mice after 50 days treatment A-B: (A= Control; B= Disease control

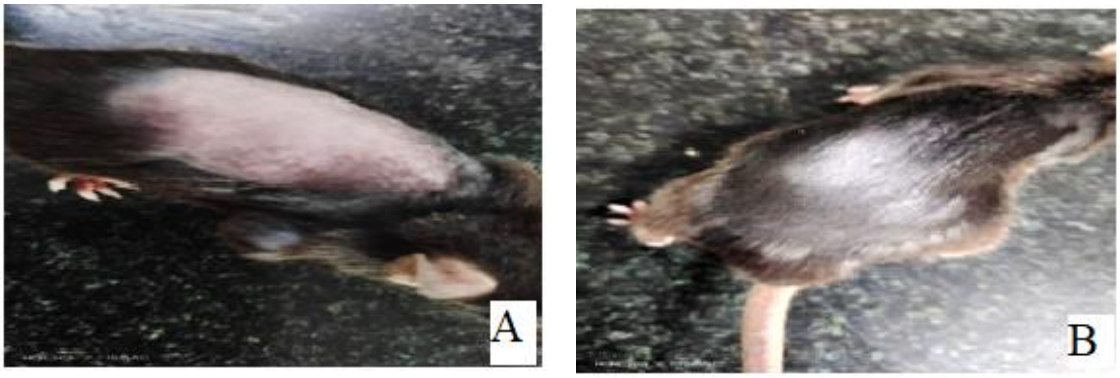


Figure 39: Photographs of C57 mice after 50 days ointment treatment A-B: (A= Chloroform extract ointment; B= Ethyl acetate extracts ointment

The evaluation of skin depigmentation utilized a scoring system (refer to Table 46), encompassing assessments of hair color, skin color, and hair growth.

Table 46: Scoring criteria for the skin and hair depigmentation model

Skin color	Hair color
Normally colored	Black Normal
Grey colour	White colour (showing depigmentation)
White (showing depigmentation)	blend of black and white 1:1 coloured hair
blend of black and white 1:1 coloured skin	Intense black colour
Black colour (showing induced pigmentation)	Intense white colour

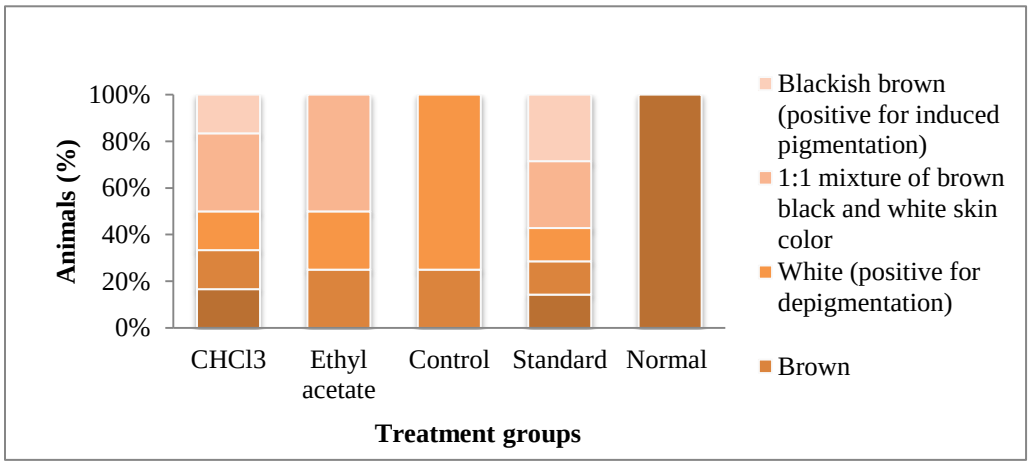


Figure 40: Scoring parameters for depigmentation skin

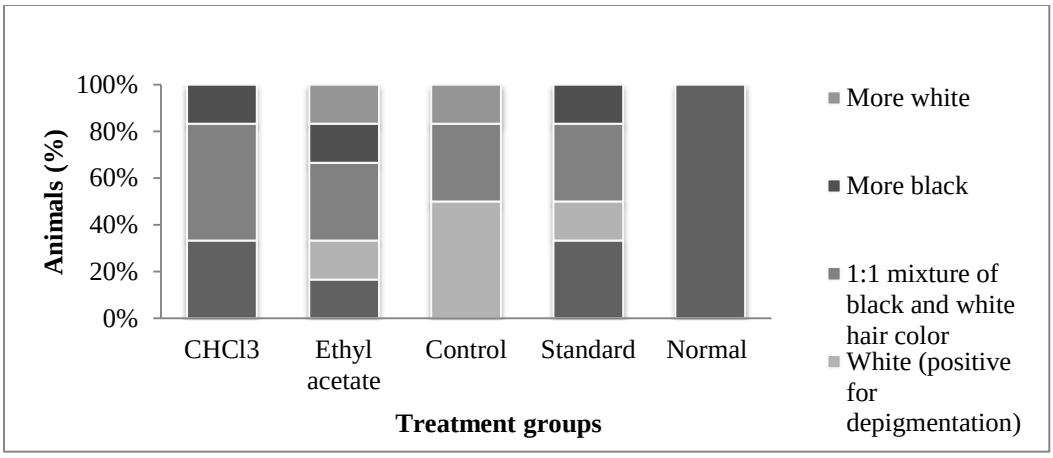


Figure 41: Scoring parameters for depigmentation hair colour.

8.4 Anti-oxidant studies of developed ointments

For the evaluation of anti-oxidant of developed formulation, the ointment was solubilized in dimethyl sulfoxide at concentration of 10 mg/ml (Rhimi *et al*., 2019).

8.4.1 Results of DPPH radical scavenging activity

These results suggest that both the chloroform and ethyl acetate extract ointments possess anti-oxidant activity, with the ethyl acetate extract ointment demonstrating a slightly higher anti-oxidant activity compared to the chloroform extract ointment as indicated in table 47. The percentages results with standard deviation (S.D) indicate the level of anti-oxidant activity exhibited by the ointment being tested. The standard deviation values indicate the variability or precision of the measurements.

Table 47: DPPH radical-scavenging activity of *Heracleum candicans* ointment

Ointment (10%)	DPPH %
Chloroform extract	68.72 ± 0.68^{b}
Ethyl acetate extract	81.56 ± 0.41^{a}

Where a, b indicate the significance of values reported during the evaluation on the basis of various P values.

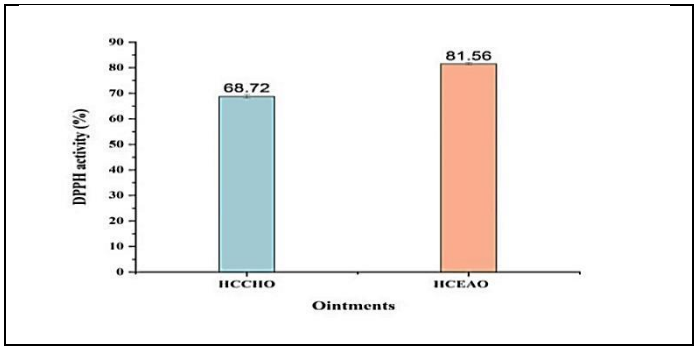


Figure 42: DPPH radical-scavenging activity of *Heracleum candicans* ointment; HCEAO= *H. candicans* ethyl acetate extract ointment, HCCHO= *H. candicans* chloroform extract ointment.

8.4.2 Results of Reducing Power activity

These results suggest that both the chloroform and ethyl acetate extract ointments possess reducing power potential, with the chloroform extract ointment exhibiting a higher RP value compared to the ethyl acetate extract ointment as indicated in table 48. The standard deviation values indicate the variability or precision of the measurements.

Table 48: Reducing power activity of *Heracleum candicans* ointment

Ointment (10%)	RP
Chloroform extract	0.53 ± 0.04^{a}
Ethyl acetate extract	0.36 ± 0.01^{b}

Where a, b indicate the significance of values reported during the evaluation on the basis of various P values.

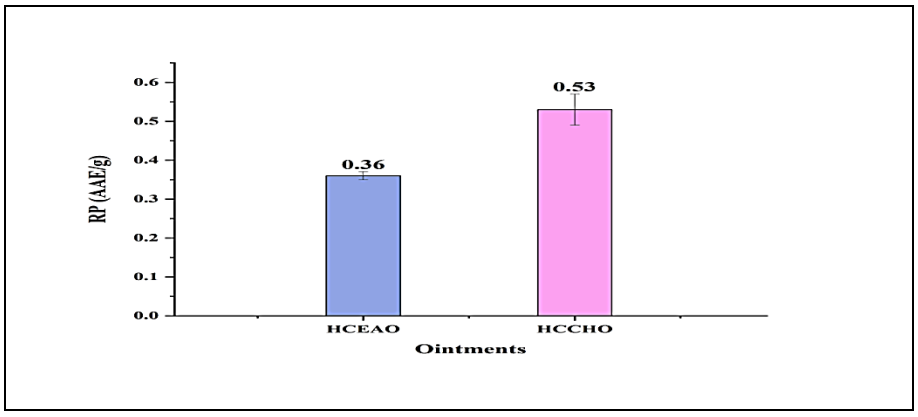


Figure 43: Reducing power activity (RP) of *Heracleum candicans* ointment; HCEAO= *H. candicans* ethyl acetate extract ointment, HCCHO= *H. candicans* chloroform extract ointment

8.4.3 Results of Total flavonoid content (mg QE/g)

The quantification of TFC is denoted in micrograms of quercetin equivalents (or an alternative standard flavonoid) per milliters of the specimen *(µg QE/mL).* Quercetin serves as a frequently employed reference standard in these assessments. These results suggest that the chloroform extract ointment of *Heracleum candicans* contains a higher total flavonoid content compared to the ethyl acetate extract ointment as indicated in table 49. Flavonoids are known for their anti-oxidant activity, so a higher TFC may indicate potentially greater anti-oxidant properties in the chloroform extract ointment. The standard deviation values indicate the variability or precision of the measurements.

Table 49: Total flavonoid content of *Heracleum candicans* ointment

Ointment (10%)	TFC
Chloroform extract	52.6±0.91[a]
Ethyl acetate extract	30.45±0.56[b]

Where a, b indicate the significance of values reported during the evaluation on the basis of various P values.

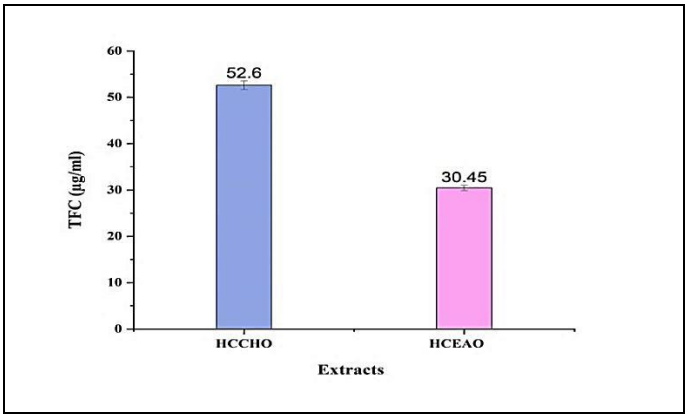


Figure 44: Total flavonoid content (TFC) of *Heracleum candicans* ointment; HCEAO= *H. candicans* ethyl acetate extract ointment, HCCHO= *H. candicans* chloroform extract ointment

8.4.4 Results of Total phenolic content

The results are expressed in mg of GAE per gm. of Gallic acid**.**These results suggest that the ethyl acetate extract ointment of *Heracleum candicans* contains a higher total phenolic content compared to the chloroform extract ointment as indicated in table 50. Phenolic compounds, like flavonoids, are known for their anti-oxidant properties, so a higher TPC may indicate potentially greater anti-oxidant activity in the ethyl acetate extract ointment. The standard deviation values indicate the variability or precision of the measurements.

Table 50: Total phenolic content of *Heracleum candicans* ointment

Ointment (10%)	TPC
Chloroform extract	95.30 ± 0.86^{b}
Ethyl acetate extract	101.62 ± 1.15^{a}

Where a, b indicate the significance of values reported during the evaluation on the basis of various P values.

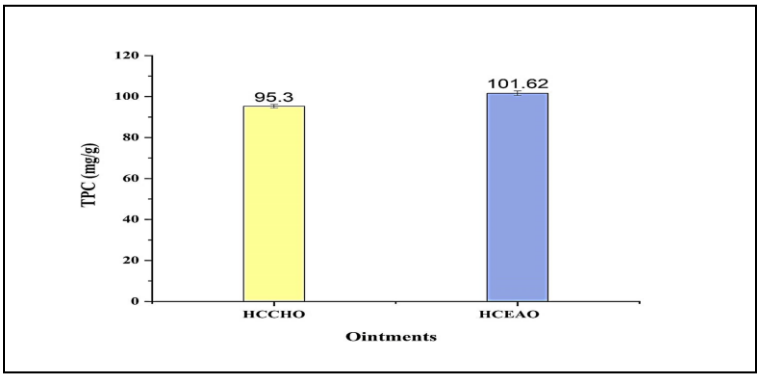


Figure 45: Total phenolic content (TPC) of *Heracleum candicans* ointment; HCEAO= *H. candicans* ethyl acetate extract ointment, HCCHO = *H. candicans* chloroform extract ointment.

8.4.5 Results of Lipid peroxidation inhibition

Lipid peroxidation is a process where free radicals steal electrons from the lipids in cell membranes, resulting in cell damage. Inhibition of this process is often associated with anti-oxidant activity. For the Chloroform extract (HCCHO), the ILP percentage is

43.78% with a standard deviation (S.D) of 0.56. For the ethyl acetate extract (HCEAO), the ILP percentage is 59.43% with a standard deviation (S.D) of 0.91. These results suggest that the ethyl acetate extract ointment of *Heracleum candicans* exhibits a higher lipid peroxidation inhibition percentage compared to the chloroform extract ointment as shown in table 51. Higher ILP percentages indicate greater inhibition of lipid peroxidation, which is associated with stronger anti-oxidant activity. The standard deviation values indicate the variability or precision of the measurements.

Formula: % Inhibition = [1- (A of sample/A of control)] × 100

Table 51: Lipid peroxidation inhibition of *Heracleum candicans* ointment

Ointment (10%)	ILP(%)
Chloroform extract	43.78 ± 0.56^{b}
Ethyl acetate extract	59.43 ± 0.91^{a}

Where a, b indicate the significance of values reported during the evaluation on the basis of various P values.

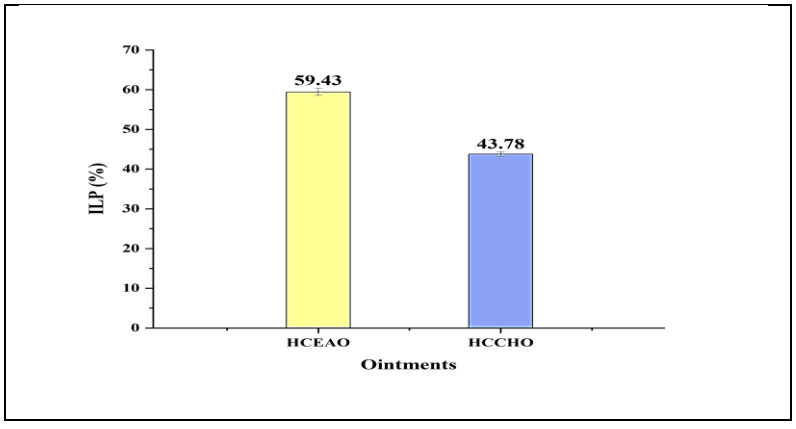


Figure 46: Lipid peroxidation inhibition of *Heracleum candicans* ointment; HCEAO= *H. candicans* ethyl acetate extract ointment, HCCHO= *H. candicans* chloroform extract ointment, ILP= Lipid peroxidase inhibition

8.5 Conclusion

Melanin synthesis plays a crucial part in human physiological processes, primarily in protecting from the hazardous properties of ultraviolet radiations on skin (Zaidi, 2017).

Melanin functions as a key defense mechanism against skin damage induced by solar UV irradiation. The absence of melanin is closely associated with the progression of dermal conditions like vitiligo (Kaidbey *et al*., 1979). The process of melanogenesis encompasses various stages, including melanin synthesis, transport, and release. A reduction in tyrosinase activity within melanocyte bulbs is linked to the loss of melanin in hair shafts (Zaidi, 2017). The results of the anti-oxidant indicate that the chloroform extract when formulated in an ointment has better anti-oxidant activity than the ethyl acetate extract ointment. The results are inconformity with the anti-oxidant activity of extracts. It can be concluded, that chloroform extract of the plant has significant anti-vitiligo potential as compared to ethyl acetate extract of the plant.

SUMMARY OF THESIS

Vitiligo is a prevalent skin disorder affecting 1–2% of the global population, characterized by the depletion of dermal pigment-producing cells, specifically melanocytes. The pigment-producing cells in individuals with vitiligo are more susceptible to exogenous oxidative stresses, leading to increased amounts of reactive oxygen species, and elevated endoplasmic reticulum stress. Various treatment options have been explored, including Ayurveda, surgery, homeopathy, and conventional medicine. Among these, herbal medicine has gained significant attention, with a focus on immunomodulatory, anti-inflammatory, and anti-oxidant-rich formulations.

One such herbal candidate under investigation is *Heracleum candicans*, a less explored Himalayan medicinal plant traditionally used against Leucoderma. Some compounds, like furanocoumarins like heraclenin, 8-geranyloxypsoralen, imperatorin, candicanin, bergapten, isopimpinellin, pimpinellin, and sphondinin, were found in the plant material through preliminary phytochemical analysis. To help with the correct identification of this medicinal plant, pharmacognostic studies found prismatic calcium oxalate crystals, anomocytic stomata, and two types of covering trichomes.

The research illustrates the analysis of the medicinal, physical, and chemical characteristics of the above-ground and underground parts of *H. candicans*. The findings will aid in accurately identifying this plant for future research purposes. The pharmacognostic analysis represents a fundamental and trusted method used for recognizing and evaluating the safety and authenticity of raw medicinal substances. Upon morphological examination, both aerial, as well as root sections, portions presented a white-felted inner surface beneath the big pinnately segmented leaves, that possessed a distinctive appearance and emanated a subtle odor.

Pinnate basal and lower leaves were in 2-3 pairs, ovate-oblong, 4–13 cm long, 3-6 cm wide, pinnatified, silvery abaxially, apex obtuse, white tomentose, and margins serrate. Sessile-reduced upper leaves, three lobed on expanded sheaths Peduncles 15–28

cm long; bracts 1-3, linear, pubescent; caducous; rays 15–30, unequal, 3–10 cm long; umbellules 20–25-flowered. Flowers are white; the outer petals of the flowers are larger and bilobed. The stem is solitary and branched. Minute calyx teeth, along with white petals, outer flowers of umbels radiant, Fruit is obovoid, 6–12 mm long, 4–7 mm wide, and glabrous when mature.

Upon microscopic evaluation, a few pieces of the lamina were seen when the aerial part of *H. candicans* was looked at under a powder microscope. The upper epidermis consisted of polygonal cells with mildly undulating walls, braided-like structures, and unevenly expanded regions. Cells revealed enhanced elongation in sites above the veins. It included many single-celled and non-woody trichomes with elongated cells. The fine powder of the aerial parts of *H. candicans* comprised lots of tiny crystals of calcium oxalate, mostly prismatic in shape and varied in size. Additional identifiable tissues comprise lignified fibers, a limited number of pits, as well as reticulate and spiral vessels. The stomata observed in the parenchymatous tissue of the aerial powder were mainly of the anomocytic form.

The plant notably consists of chemically polar substances, as indicated by the elevated percentages of alcoholic and water extractive values, obtained using cold, hot, and repeated extraction methods. The pH values of 1% and 10% solutions of aerial portion turned out as 6.24 and 6.00, respectively. These findings imply the existence of acidic components in the plant. The initial investigation of plant phytochemicals suggested the existence of several phytochemicals such as carbohydrates, alkaloids, flavonoids, tannins, phenolic compounds, and terpenoids. In addition, an approach for thin-layer chromatography (TLC) of the plant was also established. It was noticed that nearly all of the extracted material used in the TLC experiments included a significant number of phytoconstituents.

The research also demonstrated that various extracts from the aerial parts of *H. candicans* plant possess potent anti-oxidant properties. It was found that ethyl acetate had the most total phenolic content, followed by chloroform and petroleum ether. The most powerful DPPH radical-scavenging compound was found to be ethyl acetate. This shows

that DPPH radical scavenging and anti-oxidant potential are related in a good way. The chloroform extract displayed the highest total flavonoid content. Additionally, the lipid peroxidation inhibition assay ranked ethyl acetate as the most effective extract.

Further studies focused on cytotoxicity against B16F10 cell lines, revealing moderate cytotoxic potential in psoralen, xanthotoxin, chloroform extract, ethyl acetate extract, methanolic extract, and hydroalcoholic extract. The petroleum ether extract exhibited effective cytotoxicity. Tyrosinase activity studies demonstrated dose-dependent inhibition for psoralen and xanthotoxin, with notable inhibition in the hydroalcoholic extract.

In vitro melanin secretion studies in B16F10 cells indicated concentration-dependent effects of psoralen, xanthotoxin, and various *H. candicans* extracts. Psoralen exhibited a stimulatory effect on melanogenesis, surpassing xanthotoxin. Notably, chloroform extract induced a significant increase in melanin content, followed by the ethyl acetate extract of the plant.

To assess *in vivo* anti-vitiligo properties, a chloroform extract and ethyl acetate from *H. candicans*, containing psoralens and xanthotoxin, was formulated into an ointment. Topical application of a chloroform extract and ethyl acetate ointment for 50 days on C57 mice led to a gradual disappearance of vitiligo, supported by reduced N-acetyl-d-glucosamine levels compared to the monobenzone-treated group. Histological assessment of the skin of the C57 mice revealed the level of depigmentation was lower in the chloroform extract-treated groups as compared to the ethyl acetate-treated and monobenzone-treated groups. The literature suggests that coumarin-containing plants, like *H. candicans*, are traditionally used for skin repigmentation.

Herbal formulations developed for topical application were analyzed for various attributes: color, odor, loss on drying, non-irritancy, pH, diffusion study, spreadability, extrudability, solubility, consistency, washability, and viscosity. The F2 formulations of the chloroform extract show promising characteristics. Further stability studies to assess the stability of herbal ointment combinations using various physicochemical metrics were also conducted for the developed formulation. The results of the stability studies implied

that the F2 formulation of the chloroform extract was stable under the given set of temperature conditions.

In conclusion, the comprehensive study on extracts from the aerial parts of *H. candicans* highlights that the plant is safe and has good potential for vitiligo treatment. The plant's rich phytochemical profile, anti-oxidant activity, cytotoxic potential, and *in vivo* anti-vitiligo effects support its traditional use. Both of these promising activities found in the plant are directly involved in the pathogenesis of vitiligo. The F2 formulation of the chloroform extract, exhibiting stability and promising attributes, holds promise for further development in topical applications and can indeed be of great help to individuals who are socially stigmatized by this malady due to the lack of credible treatment options on the market for its mitigation and prevention.

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APPENDICES

LIST OF AWARDS, PATENTS, PUBLICATIONS, PRESENTATIONS AND WORKSHOPS

Patents

- Dar, NA Tewari D, Bhat ZA, A topical Herbal Formulation for Therapeutic Management of Vitiligo, filed under application no.: 202011042085, October 2020.

Copyrights

- Copyright for the research topic Himalayan *Heracleum candicans* ointment For anti-vitiligo activity has been filed under application no.: 10979, October 2023.

Publications from my Ph.D. research work

- Dar, N. A., Raja, W. Y., Tewari, D., & Bhat, Z. A. (2022). Pharmacognostic study of roots and aerial parts of less explored Heracleum candicans Wall. ex DC. from Betaab Valley, Pahalgam, Kashmir, India. IJNPR Vol.13(3) [September 2022], https://doi.org/10.56042/ijnpr.v13i3.40840

Presentations

- Dar NA, Jan R, Raja WY, Bhat ZA, presented through oral presentation a paper on "*An attempt to cultivate Heracleum candicans from its seeds along with GC-MS analysis".* 2nd International conference on "*Advances in Biopolymers" and Workshop on "Fermented Foods & Gut Health*" organized by the department of Food Sciences and Technology, University of Kashmir, November 8-9, 2021.
- Dar NA, participated in an international conference on " *Emerging Innovations and Entrepreneurship in Biotechnology (EIEBT 2021)"*, organized by

Department of Biotechnology, Manonmaniam Sundaranar University, Tirunelveli on 21-22 December 2021.

- Dar NA, Attended a three days international conference on *"Ecosystem Restoration and Environmental Sustainability, Concerns and Alternatives*" at University of Rajasthan, Jaipur on October 26-28, 2021.
- Dar NA, participated in an national conference on "*Opportunities and Challenges in Clinical Research and Pharmacovigilance in India*" organized by Seven Hills College of Pharmacy,Tirupati, Andhra Pradesh on 17-18th September 2021.
- Dar NA, Tewari D, Raja WY, Bhar ZA, presented a paper on Chemical Characterization and Antimicrobial Potential of Less Explored Heracleum candicans Wall Ex. DC" during International conference on "Food Nutrition, Health and Lifestyle" 2022 organized by the Institute for Knowledge Management, Sri Lanka on 15-16th July 2022.
- Nissar Ahmad, Participated in an international conference on " India-Russia domestic and bilateral cooperation"
- Nissar Ahmad, Participated in an international conference on "Natural Products and Human Health and Challenges in Present Scenario"

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Pharmacognostic study of roots and aerial parts of less explored *Heracleum candicans* Wall. ex DC.from Betaab Valley, Pahalgam, Kashmir, India

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Plant derived products have sparked considerable attention due to their versatile applications. The plants are among the richest bio-resource of drugs for traditional and modern medicines and leads for synthetic drug development and various pharmaceutical intermediates. *Heracleum candicans* Wall. ex DC. is reported as a medicinal herb of Apiaceae family. It is also known as White-leaf Hogweed and traditionally reported to be effective in various disease conditions like skin diseases, sunburn, and external tumours. The present work deals with the pharmacognostic evaluations of root and aerial parts of *H. candicans*. Macroscopic and microscopic analysis was carried out along with various physicochemical analysis, phytochemical screening as well as thin layer chromatography studies. Moreover, the presence of heavy toxic metals by ICP-OES method was also evaluated. The pharmacognostic studies of *H. candicans* showed the presence of prismatic calcium oxalate crystals, anomocytic stomata, reticulate and spiral xylem vessels along with lignified fibres with two types of covering trichomes. Preliminary phytochemical studies revealed the occurrence of carbohydrates, flavonoids, phenols, tannins, saponins, phytosterols, diterpenes, coumarins, cardiac glycosides, fats and oils. The estimation of various physicochemical constants could be beneficial in determining various quality control standards for crude drug. The findings from this study would be beneficial for the identification of *H. candicans*.

Keywords: Flavonoids, *Heracleum candicans*, Microscopy, Phenols, Quality control.

IPC code; Int. cl. (2021.01)- A61K 36/00, A61K 125/00, A61K 127/00

Introduction

The search for medicines for various disease conditions from natural resources has been performed since ancient times. Various knowledge exchange, memorials, and written documentation are evidence for this. Around two-thirds of novel, medications were directly or indirectly developed from plants. Hence, comprehensive studies on phytoconstituents from traditional medicines using phytochemical, pharmacological, and analytical methods are the need of the hour[1]. The standardization of crude drugs is of paramount importance that begins with the collection of botanical materials and ends with their packaging and usage as medicine. Microscopy, macroscopy, physicochemical characteristics, extractive values, fluorescence analysis, and heavy metal analysis are utilized to set pharmacognostical standards. These characteristics, in turn, can serve to ensure the drug's quality[2]. Amongst the family of Angiosperms, Apiaceae or Umbelliferae is the largest family and recognized as diverse group of species of medicinal value[3]. The family comprises of about 450 genera and 3,700 species worldwide[4]. In India, of the total 186 species (representing 55 genera) of this family, 150 (80.6%) species representing 45 genera (81.8%) are found in the Himalayan region[5].

Heracleum is a derivative of Latin word, *Herâclêus* or belonging to *Hercules* (itself derived from the Greek) means 'glory of Hera'. Around 125 *Heracleum* species are present globally with the Sino-Himalayan and Caucasus region as the major centres of diversity[6-8]. Genus *Heracleum* (family Umbelliferae) or 'Hogweed' is a perennial plant spread in Asia, Europe, North America and Abyssinia[9]. This genus is widely distributed in Asia[10]

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Certificate

This is to certify that the project proposal No. IAEC-2023-01-02, entitled **"Development, Evaluation and Characterization of Novel Anti-Vitiligo ointment from Himalayan *Heracleum candicans Wall.ex* DC."** submitted by **Nisar Ahmad Dar** has been approved/~~recommended~~ by the IAEC of University of Kashmir, in its meeting held on **24-01-2023** and **24, Swiss mice/C57 BL/6** (Number and Species of animals) have been sanctioned under this proposal for a duration of twelve months.

Authorized by	Name	Signature	Date
Chairperson: -	*Prof.Nahida Tabassum*	.....	24-01-2023
Member Secretary: -	*Dr.Mohammed Iqbal Zargar*	.....	24-01-2023
Main Nominee of CPCSEA: -	*Dr.Mujeeb Ur Rehman Fazili*	.....	24/01/2023

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CENTRE FOR BIODIVERSITY & TAXONOMY
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(NAAC Accredited grade "A")

No: KASH/vouch/speci/19 Dated: 22/10/19

CERTIFICATE

This is to certify that Mr Nissar Ahmad Dar pursuing Ph. D in the Department of Pharmaceuticals Sciences,(Pharmacogonsy) Lovely Professional University Phagwara Jalander Punjab submitted the specimen in our **KASH Herbarium at Kashmir University** under Voucher specimen number. The plant specimen have been identified by undersigned at Centre for Biodiversity and Taxonomy, University of Kashmir on the basis of morphological characters. The following voucher specimen number was issued as under:

***Heracleum candicans* Wall. ex DC Voucher Specimen No.2847-(KASH) Herbarium, Centre for Biodiversity and Taxonomy, University of Kashmir, 21-09-2019,Ferozpora-Tangmarg.**

Akhtar H. Malik

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Dated: 23 Sep 2019

Nissar Ahmad Dar
Registration Number: 41800248
Programme Name: Doctor of Philosophy (Pharmacognosy)

Subject: Letter of Candidacy for Ph.D.

Dear Candidate,

We are very pleased to inform you that the Department Doctoral Board has approved your candidacy for the Ph.D. Programme on 23 Sep 2019 by accepting your research proposal entitled: "DEVELOPMENT, EVALUATION AND CHARACTERIZATION OF NOVEL ANTI-VITILIGO OINTMENT FROM HIMALAYAN Heracleum candicans Wall. ex DC"

As a Ph.D. candidate you are required to abide by the conditions, rules and regulations laid down for Ph.D. Programme of the University, and amendments, if any, made from time to time.

We wish you the very best!!

In case you have any query related to your programme, please contact Centre of Research Degree Programmes.

Head

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Certificate of Participation

Mr. Nissar Ahmad

This is to certify that

participated in the Second Indo-Russian Young Scholars International E-Conference on "India-Russia domestic and bilateral cooperation, in the Mark of 50th Anniversary of the treaty of peace, friendship and cooperation between India and Soviet Union organized by Eurasian Foundation, New Delhi, India, in collaboration with Centre for Indian Studies of Institute of Oriental Studies, RAS, Moscow, Russia, Centre for Central Eurasian Studies, University of Mumbai, India, Centre for Third Voice Studies, India and Russian Centre for Science and Culture, New Delhi, India on 16-18 September, 2021. We fully appreciate his/her contribution in the International conference.

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Certificate of Participation

This is to certify that

Nissar Ahmad

has attended the **2nd ICABP** held at University of Kashmir, Hazratbal, Srinagar, India, from November 8 - 9, 2021 and presented a paper through an oral/~~poster~~ mode

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DEPARTMENT OF BIOTECHNOLOGY

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

(REACCREDIATED WITH 'A' GRADE BY NAAC)

PARTICIPATION CERTIFICATE

This is to certify that

Nissar Ahmad Dar

has participated in the International conference on "Emerging Innovations and Entrepreneurship in Biotechnology(EIEBT-2021)" organized by the Department of Biotechnology, Manonmaniam Sundaranar University on 21st and 22nd December, 2021.

DBT - MSU

CONVENOR


SEVEN HILLS COLLEGE OF PHARMACY
(AUTONOMOUS)
Venkatramapuram, RC Puram (Mandal),Tirupati-517561,Chittoor(Dist.,), Andhra Pradesh, India.
Accredited "A" Grade by NAAC, Bangalore & NBA(U.G), New Delhi.
AICTE Sponsored NATIONAL ONLINE CONFERENCE
On
"Opportunities and Challenges in Clinical Research and Pharmacovigilance in India"
CERTIFICATE OF PARTICIPATION
This is to certify that Prof/Dr/Mr/Mrs/Ms Mr. Nissar Ahmad of
Lovely Professional University has participated in the
AICTE Sponsored National Online Conference "CR & PV-21" on September 17 & 18, 2021 at Seven Hills College of Pharmacy
(Autonomous), Venkatramapuram, Tirupati-517561, Andhra Pradesh, India.
Parton
Dr. M.Niranjan Babu
Co-Parton
Dr. K. Umamaheshwara Rao
Convenor
Dr. B. Jyothi
CO-convenor
Mrs. B. Sivagami

The 4th International Conference on Food, Nutrition, Health and Lifestyle 2022 (Nutricon 2022)

"Future & Emerging Trends for 2022 in Nutrition, Food & Health"

15th – 16th July 2022 | Virtual Conference

Author	:	Dar NA
Co - Author	:	Tewari D, Raja WY and Bhat ZA
Paper ID	:	NUTRICON 2022 A 166
Institute	:	University of Kashmir, India
Notification Date	:	04th May 2022

NOTIFICATION OF ABSTRACT ACCEPTANCE – NUTRICON 2022

Dear Ahmad Dar,

Congratulations! Your abstract titled **"CHEMICAL CHARACTERIZATION AND ANTIMICROBIAL POTENTIAL OF LESS EXPLORED HERACLEUM CANDICANS WALL. EX DC. FROM PAHALGAM, KASHMIR, INDIA'S NORTHERN HIMALAYAN REGION"** has been accepted for Oral Presentation subjected to a double-blind peer reviewing process conducted by the Scientific Reviewing Committee of the 4th International Conference on Food, Nutrition, Health and Lifestyle 2022 (NUTRICON 2022). On behalf of the Conference Organizing Committee, I would like to formally invite you to attend NUTRICON 2022 to present your paper on 15th – 16th July 2022 in the Virtual Platform.

NUTRICON 2022 is hosted by Universitas Hasanuddin, Indonesia. The Conference is organized by The International Institute of Knowledge Management (TIIKM) Sri Lanka in collaboration with University of Brawijaya, Indonesia, University of Muhammadiyah MAKASSAR, Indonesia, Universiti Malaysia Sabah, Malaysia, The State Islamic University of Sunan Kalijaga, Indonesia, Universitas Sumatera Utara, Indonesia and National Institute of Food Technology Entrepreneurship and Management, India as Academic Partners of the Conference.

The Conference offers an enriching opportunity to deepen knowledge of global food and nutrition along with the theoretical, policy, and personal dimensions of food and nutrition scholar-activism worldwide, engaging with intersecting fields of food and nutrition Studies and the many disciplines that constitute food and nutrition —all toward building collaborative partnerships, offering publishing opportunities, and enacting visions of a more equitable and liberal world.

All Accepted Abstracts of the registered participants for the NUTRICON 2022 will be published in the Abstract Book with an associated ISBN 978-624-5746-11-8.

NUTRICON 2022 will be supported by high-ranked internationally peer-reviewed journals which are indexed in SCOPUS and other indexing databases. The registered participants are privileged to submit their full papers

Conference Secretariat,
NUTRICON 2022,
The International Institute of Knowledge Management.
No. 531/18 | Kotte Road | Pita Kotte | Sri Lanka.
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International E-Conference
on Current Science and Technology for Sustainable Society

aurora

Certificate of Participation

This is to certify that

Dr./Mr./Ms. **Nissar Ahmad**

Lovely Professional University

has participated in the **International E-Conference on "Current Science and Technology for Sustainable Society"** organized by Aurora's Degree & PG College, Hyderabad and A.V. Kanthamma College for Women, Hassan on 27th & 28th November 2020.

Dr. K.G. Suresh Kumar, Patron
Dr. B.S. Ravi Kumar, Organizing Secretary
Dr. S. Ravi Kiran, Organizing Secretary
Dr. Viswanadham Bulusu, Patron

Aurora's Degree & PG College, Chikkadpally, Hyderabad - 500 020, Tel: 040 2766 2668, URL: www.adc.edu.in
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DEPARTMENT OF GEOGRAPHY, FACULTY OF EARTH SCIENCES
MOHANLAL SUKHADIA UNIVERSITY, UDAIPUR - 313001 (RAJ.) INDIA

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Certificate of Participation

This is to certify that Prof./Dr./Ms./Mr. Nissar Ahmad of Lovely Professional University has participated in IGU India International Conference (Virtual), 2020 on "Global to Local Sustainability and Future Earth" from December 18th -20th, 2020 organized by the Department of Geography, MLSU, Udaipur (Raj.), India.

Prof. Sadhana Kothari
Convener

Dr. Bhanwar Vishvendra Raj Singh
Organizing Secretary

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CERTIFICATE

This is to certify that *Nissar Ahmad*, *Research Scholar*, *Lovely Professional University* has participated in the Science Academies' sponsored Virtual Lecture Workshop on Emerging Trends of Computational methods in Drug Development organized by Department of Physics, NERIST during 6-7 November, 2020.

Th. Gomti Devi		(H. S. Yadav)
Dr.Th.Gomti Devi	**Prof. Tado Karlo**	**Prof. H.S. Yadav**
Coordinator	**HOD(Physics)**	**Director, NERIST**

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CERTIFICATE OF PARTICIPATION

This is to certify that Nissar Ahmad, Research Scholar, Department of Pharmacy, LOVELY PROFESSIONAL UNIVERSITY has participated in a one week Faculty Development Program (Online) on "**Recent Trends in Materials Science (RTMS - 2020)**" organized by Physics Division, Department of Basic Science and Humanities, GMR Institute of Technology, Rajam during 12th - 16th October, 2020.

Dr. M. Lakshmiprasad	Dr. R.L.Naidu	Dr. J.Raja Murugadoss	Dr. C.L.V.R.S.V. Prasad
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