Chapter

Obtaining Cell Cultures of Medicinal Plants

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Abstract

In vitro propagation of medicinal plants has been incorporated into producing healthy plants that are beneficial to humanity. Some basic principles and factors tend to influence the cultivation process, thus, causing this method of plant propagation to be adapted owing to the importance and benefits surrounding this method. The main objective of this research work was to obtain cell cultures of medicinal plants of *Cichorium intybus, Stevia rebaudiana Bertoni, Monarda citriodora, and Rhodiola krylovii*. In obtaining the cell cultures of these medicinal plants, some steps need to be followed. In this research, the effect of different methods of sterilisation/cultivation of plant seeds and explants were evaluated using two different media compositions, observable differences between sterile and non-sterile plant seedlings of *C. intybus, Monarda citriodora, and Rhodiola krylovii.* The effect of growth regulator (Kinetin) and non-growth regulator (Kinetin) on the cell cultures was observed in solid and liquid media; the dry and wet weight was determined for a callus of Chicory grown in cell suspension culture. All results were presented on tables and charts.

Keywords: medicinal plants, callus, solid media, liquid media, suspension culture, *Cichorium intybus*, *Stevia rebaudiana Bertoni*, *Monarda citriodora*, *Rhodiola krylovii*

1. Introduction

Plant cell cultivation based on the *in vitro* process is not general for medicinal species. Therefore, many questions are yet to be dealt with regarding the cultivation and quality assessment of the plants produced via the *in vitro* method of cultivating these plant materials, such as seeds, callus, seedlings, and hairy roots. Plant cell culture is a technology that investigates some of the conditions that promote cell division and other growth factors in *in vitro* conditions, and it is considered a valuable tool in both primary and applied studies and commercial applications [1, 2]. It has been demonstrated in previous studies that factors, such as irrigation and nutrient status, affect the chemical profile and composition of plants. Since the understanding is limited of the mechanisms and activity of herbal formulations to combat disease, quality assessment is often reduced to quantifying one or a few compounds. Herbal formulations are, however, very complex, and only a few selected compounds, can be optimal to determine the changes in the chemical profile and composition of cultivated plants [3].

Medicinal plant cultivation employs basic materials, such as substrate, small mineral elements, water, and light, to grow without stress compared to animal rearing. Environmental conditions and climatic conditions can harm plants growing in an outdoor setting. Despite the changes caused by environmental factors, such as elevated temperature, high humidity, and others, thus, to avoid repeatability and uniformity across the experiment, it is necessary to define and control specific growth conditions by growing plants indoors for research [4].

The progressiveness of subsistence culturing of plants used for commercial trading in the area of medicinal plants has assumed rise to an increase in the rate at which these medicinal plants are harvested from wild habitats [5]. When harvested to an extent, every plant can be exposed to annihilation, although medicinal tree species are most vulnerable to harvesting as they are slow-growing, slow- reproducing and many have specific habitat requirements that limit their distribution [6]. Thus, plants withering owing to harvesting are, therefore, not readily replaced. For centuries the sustainable use of medicinal plants was facilitated by several indirect control methods and some intentional management practices. Some of these practices became unused as development and alteration in traditional healing practices were experienced.

Plant cell culture is a technique that investigates the conditions that influence cell division and genetic regeneration *in vitro* propagation, and it is considered an essential tool in both applied and fundamental studies, additionally as in commercial application [1, 2]. Presently, the facilities for *in vitro* cell cultures are found to be applicable in each plant biology laboratory as a helpful tool for various purposes since tissue culture has turned into a fundamental asset for modern biotechnology, from the critical biochemical aspects to the massive propagation of selected individuals. There are five main areas where *in vitro* cell cultures are being currently applied and can be recognised: As a model system for essential plant cell physiology aspects, generation of genetic modified fertile individuals, large-scale propagation of elite materials, preservation of imperilled plant species, and metabolic engineering of fine chemicals [7].

In addition, *in vitro* culture is a technique that involves the replication of new cells, tissues, and organs derived wholly from the mitotic cell division, consequently generating cloned cells, tissues, and individuals, viz., with identical genetics to the mother plant. Application of *in vitro* culture techniques to effectively produce secondary metabolites, basically plant-derived medicinal compounds, has its main advantages as follows:

1. Decrease in environment interference due to conditions controlled in the *in vitro* culturing chamber.

2. The contingency of having greater control over PDMCs production. Whereby a step-by-step protocol was formed to speed up fresh biomass, accumulation and increase PDMCs concentration in the tissues,

3. The season-independent staggered production of plants-derived medicinal compounds (PDMCs) produces plant-derived medicinal compounds under sterile conditions with fewer risks of contamination by undesired toxic compounds [8].

1.1 Importance of plant tissue culture techniques

Plant tissue culture techniques can be widely used as biotechnological tools for basic and applied purposes. Thus, ranging from investigating plant developmental

processes, functional gene studies, commercial plant micro-propagation, and the generation of transgenic plants with explicit industrial and agronomical traits. Then, plant breeding and crop improvement, virus elimination from infected materials to render high-quality, healthy plant material, preservation, and conservation of germplasm of vegetatively propagated plant crops. And save plant species that are endangered by external factors, such as the environment. Additionally, plant cell and organ cultures are interested in producing secondary metabolites of industrial and pharmaceutical interest. Modern technologies, such as genome-editing ones combined with tissue culture and Agrobacterium tumefaciens infection, are currently promising alternatives for the precise genetic manipulation of attractive agronomical or industrial traits in crop plants [7].

1.2 Basic principles of plant cell culture

There are some basic principles of plant cell culture, which are as follows: (1) Select an appropriate explant from a healthy and vigorous plant, (2) eradicate microbial infection of an explant from the surface, (3) inoculate the explant in an adequate culture medium, and (4) provide the explant in culture with the suitable controlled ecological conditions. In the case of *in vitro* regenerated plants, they are subjected to acclimatisation process in the greenhouse before the transference to *ex vitro* conditions. *In vitro* clonal propagation is one of the most current extended commercial applications of tissue culture [9]. These principles are contingent on the part of the plant that is cultured; we can refer to them as cell culture (gametic cells, cell suspension, and protoplast culture), tissue culture (callus and differentiated tissues), and organ culture (any organ, such as zygotic embryos, roots, shoots, and anthers, among others). Each type of culture is used for different basic biotechnological applications [9].

While demand for a consecutive increase in the supply of medicinal herbs to accelerate the evacuation of natural and artificial resources, enhancing various medicinal plants in domestication, adaptation, and cultivation can emerge as an essential strategy for facing the increasing request. Overall, the tendency is towards a more significant proportion of cultivated material in all countries [10]. Most of the national and international companies worldwide, such as over-the-counter markets, the mass and niche markets, and many herb companies, choose cultivated plants because cultivated material could be confirmed as 'organic' or 'biodynamic' [11].

1.3 Practical significance of medicinal plants

In the advanced world of medicine, medicinal plants are essential as raw materials for essential drugs, although synthetic drugs and antibiotics brought about a revolution in controlling different diseases. On the contrary, these synthetic drugs are not accessible to millions of individuals, and some research has shown that thousands of plants contain potent antioxidant compounds, especially phytochemicals and vitamins, as a result of the redox properties they possess and the effect they have to quench singlet oxygen reactive species and tendency to chelate metals [12, 13]. Those living in remote places depend on traditional healers they know and trust. The judicious utilisation of medicinal herbs can even cure deadly diseases that have long defied synthetic drugs evaluated via *in vitro* assays and by *in vivo* supplementation of human and animal models [14]. Some medicinal plants predominately have similar characteristics and components. The likes of Chicory (C. intybus), Stevia (*S. rebaudiana*

Bertoni), Bee Balm/Cambridge Scarlet (Monarda cititrodora), Rhodiola krylovii, *and Hedysarum coronarium* are found to have some standard bioactive components (see **Table 1**).

*1.3.1 Chicory (*C. intybus*)*

Medicinal plants are predominantly used as food supplements. Chicory (C. intybus) is from the Asteraceae family, a biennial/perennial herbaceous plant, and the stems and leaves are usually eaten as salads. At the same time, the roots are taken as quasi coffee after roasting since there is a similarity between the taste of Chicory to coffee taste, and it is free from caffeine [15]. In medicinal applications, the whole sectors of the Chicory plant have been used, owing to its vital bioactive compounds, such as chicoric acid, vitamins, flavonoids, phenols, sesquiterpene lactones, and fructose polymer inulin, which can act as a sweetener and pre-biotic ingredient [16, 17].

Previous studies have proposed that chicory inulin enriched with oligo-fructose improves calcium absorption and promotes bone maker in the intestines of healthy post-menopausal women [18]. Supplements of chicory help reduce iron overload and aid the proper functioning of the liver [19]. Chicory inulin has a health-enhancing mechanism potential that can disrupt the activity of gut microbiota when it is enriched, and there is a change in its composition [20]. Research has shown that increased intestinal gram-negative bacteria load in persons with diabetes can be linked to the issue of higher lipopolysaccharide (LPS) production, a structural compound in gram-negative bacteria [21]. Hence, data obtained by Landmann et al [22] indicate that chicoric acid can decrease acute alcohol-induced steatosis in mice via induction of iNOS and iNOS-dependent signalling cascades in the liver when it is altered.

*1.3.2 Stevia (*S. rebaudiana *Bertoni)*

The stevia plant has a variety of species that are rich in taste; *S. rebaudiana* is a sweetener plant that is from the genus family Asteraceae and is referred to sweet leaf, sweet weed, honey leaf, or sweet herb. It is the sweetest when compared to other

S/No	Names of Medicinal Plants Used	Characteristics	Components
1	Chicory (*Cichorium intybus*)	Asteraceae family Biennial/perennial herbaceous plant	chicoric acid, vitamins, flavonoids, phenols, sesquiterpene lactones, and fructose polymer inulin
2	Stevia (*Stevia rebaudiana Bertoni)*	Asteraceae family herbaceous plant	Stevioside (glycoside), flavanol, phenols
3	Bee Balm/Cambridge Scarlet (*Monarda citriodora*)	Herbaceous perennial/ biennial plant	Linalool, essential oils, phenols, monoterpene
4	Rhodiola krylovii (Hong Jing Tian)	Herbaceous plant	Salidroside (glucoside), tyrosol, phenols, flavanol, essential oil, monoterpene
5	*Hedysarum coronarium* L.	Herbaceous perennial/ biennial plant	Xanthones, Norisoprenoids, Flavanols, Catechins

Table 1.
Common characteristics of some medicinal plants.

Stevia species. Nevertheless, it has been identified around the globe to have a sweeter glycoside called stevioside that was the primary isolate from it; there are different other sweetness related phytochemicals, such as stevioside, rebaudioside A, B, C, D, E, and glucoside A, which has also been isolated from the leaves of *S. rebaudiana* [23, 24].

1.3.3 Bee balm/Cambridge scarlet *(*Monarda citriodora*)*

M. citriodora or *Monarda didyma*, also known as bee balm or Cambridge scarlet, is a herbaceous plant from the subgenus family Cheilyctis and from Aristatae, which originated from North America and was naturalised in Europe. These plant species have been cultivated as garden plants, food, and medicine [25].

Essential oil is a vital constituent of this plant and has been studied by many researchers due to individual species of Monarda [26].

The essential oils obtained from the leaves and flowers of *M. didyma* L. and *M. citriodora* L. cultivated in France compared to others grown in different countries had the same quantitative differences. Thus, the data collected by Collins et al. [27] show a significant component known as linalool, which was found in both flowers and leaves ranging from 64.5% to 74.2%, respectively. In the flowers, y- terpinene was 5.3% and the leaves had 0.9%. The levels of P-cymene in the flowers were 11.0%, which was higher than the composition of p-cymene in leaves at 2.1% [28].

1.3.4 Rhodiola krylovii

Rhodiola is a genus plant called Hong Jing Tian; Crassulaceae comprises over 200 species. Thus, the approximated number of species is 20, which include: *Rhodiola krylovii, Rhodiola rosea, Rhodiola alterna, Rhodiola crenulata, Rhodiola quadrifida, Rhodiola sachalinensis, Rhodiola sacra, and Rhodiola brevipetiolata* [29]. These medicinal plants are cultivated in the Himalayan belt, Tibet, China, and Mongolia. Nonetheless, they are also cultivated in Europe and North America, and these plant products are sold in the market as dietary supplements [30, 31].

Rhodiola plant varies in species, and studies carried out on varieties of Rhodiola have shown salidroside to be available in all the species of the Rhodiola genus. At the same time, rosavin (rosavin, rosin, rosarian) are compounds with a certain amount of *R. rosea* L. [32]. Rhodiola plant, particularly *R. rosea*, is often grown and used in Eastern Europe and Asia to promote physical and mental health. It is cast-off as a traditional medicine for nervous system stimulation to cure depression and fatigue, improve work output, and avoid high-altitude illness, mountain malhypoxia, and anoxia [33]. In contrast, it is used in Russia and Mongolia to treat chronic illness and weakness resulting from pathogenic infection [34]. *R. rosea* has been proven to have cardiovascular protection effects [35, 36]. In addition, the Rhodiola capsule displays anti-depressive potency in patients with depression when administered in dosages of either 0.3 or 0.6 g/day over 12 weeks. Rhodiola capsule tends to improve the eminence of life and clinical symptoms. The high doses of Rhodiola capsules are better than the lower doses [37]. Extensive efforts have been put to cultivate this plant [38].

1.3.5 H. coronarium

H. coronarium L is a perennial forage legume plant usually called French honey suckle, sulla, or sulla clover, and it is a bushy, herbaceous perennial or biennial that typically grows to 3'feet tall with a short life span. The plant is native to Northern

Africa (Algeria (N.), Morocco, Tunisia) and Europe Southwestern Europe, such as Spain. It is a plant that has been cultured as a domestic plant in the 18th century in Southern Italy and as a biennial crop for hay making, grazing, and ensiling [39]. The genus *Hedysarum L.* is approximately made up of 100 species. These are widely spread from the temperate region to boreal regions of the Northern Hemisphere. The plants of this genus occur in numerous habitats, such as deserts or seashores, alpine and arctic meadows, and stony grasslands [40, 41].

Medicinal plants can be protected through increased regulation, and the introduction of sustainable wild harvesting methods, a more viable long-term substitute is to increase domestic cultivation of medicinal plants. Cultivation also opens the possibility of using biotechnology to solve problems inherent in the production of herbal medicines. These include species misidentification, genetic and phenolic variability, variability and instability of extracts, toxic components, and contaminants. Cultivation offers the opportunity to optimise yield and achieve a uniform, high-quality product. However, the prospective cultivator of medicinal plants must make the difficult decision of which species to grow in what is a rapidly shifting and fashion-prone market [42]. Plant-specialised metabolites, also known as secondary metabolites in opposition to so-called primary ones, represent a massive reserve of bioactive compounds amenable for many human applications. Among them, polyphenols are particularly desirable in food crops due to their numerous health benefits, notably their antioxidant properties [43].

The World Health Organisation has valued that over 80% of the world's population in developing countries depend primarily on herbal medicine for basic healthcare needs [38]. However, the use of herbal medicines in developed countries is also growing, and 25% of the UK population takes herbal medicines regularly. Approximately two-thirds of the 50,000 different medicinal plant species in use are collected from the wild, and, in Europe, only 10% of medicinal species used commercially are cultivated [38]. There is concern about decreasing numbers, loss of genetic diversity, local extinctions, and habitat degradation. Well-known species threatened by wild harvesting include *Arctostaphylos uva-ursi* (bearberry), *Piper methysticum*, and *Glycyrrhiza glabra* (liquorice). Thus, between 4000 and 10,000 medicinal species might now be endangered [38, 44].

The main bioactive compounds in medicinal and aromatic plants are secondary metabolites, abiotic stress such as water deficit stress, which tends to have a more significant effect on the medicinal plants' secondary metabolites, and biologically active substances [45]. Phenolic compounds are the essential constituents in the cell defence system against free radicals in abiotic and biotic stresses and are involved in various plant processes, such as growth and reproduction [46]. Results of many studies [45–47] have shown the influences of reduced irrigation or water deficit stress on active substances of medicinal and aromatic crops.

The *in vitro* culturing of medicinal plants depends on the explant and the interaction of the medium. Hence, agar, as a conventional gelling agent, has been reported to have several drawbacks that negatively affect culture growth and differentiation. The gradual uptake of nutrients in the solid medium may lead to lower nutrient availability to the plants. Hence, a reduction in growth rate [48, 49] reported that an agar-solidified medium has lower water availability and uptake by the plants than a liquid medium. This lower uptake of nutrients could explain the lower rate of development of plantlets in solid media compared to liquid media [50]. However, the use of phytagel has not been widely reported. The liquid medium was discovered to cause more roots, nodes, and leaves in the plantlets to sprout than the solid medium; in

addition, the liquid medium is cheaper than the solid medium and more economical to use than the solid medium for potato *in vitro* micro-propagation [51].

On the contrary, the factors that affect the number of polyphenols in plant tissues depend on the age or genetic traits of the plant and many external factors, such as microorganism and pest infestation, environmental factors (temperature, humidity, and moisture), which depend on altitude and time of harvest [52–54].

1.4 Composition of culture media for growing medicinal plants

In vitro micro-propagation comes along with plantlet acclimatisation and growth in the greenhouse or the field, which is a prospect that can be incorporated in the product of secondary plant metabolites, especially in rare or endangered species, and in those difficult to propagate [55] and slow to grow [56]. The culture media is made up of minerals; micro (Mn, Zn, Cu, B, Fe, and Mo) and macronutrients (P, K, H, Mg, S, N, and Ca), and vitamins (B1 (thiamin), B6 (nicotinic acid pyridoxine)), growth hormones/regulators/stimulators (auxins, cytokinin, gibberellins, and abscisic acid), and agar (Bacto and Purified) for solid media.

1.4.1 Solid media

Sucrose 3 g, solution of iron chelate 0.5%, macronutrients 5 ml, micronutrients 0.1 ml, vitamins 0.1 ml, Kinetin 0.1 ml (c = 1 mg/ml), 1-naphthalene acetic acid (NAA) 0.4 mg/ml or 0.25 ml, agar 0.9 g, distilled water 100 ml according to Murashige and Skoog, 1962 method for media composition of 100 ml.

1.4.2 Liquid media

Sucrose 3 g, solution of iron chelate 0.5%, macronutrients 5 ml, micronutrients 0.1 ml, vitamins 0.1 ml, Kinetin 0.1 ml (c = 1 mg/ml), 1-naphthalene acetic acid (NAA) 0.1 ml, dichlorophenoxyacetic acid 0.1 ml, distilled water 100 ml according to Murashige and Skoog, 1962 method for media composition of 100 ml.

Furthermore, to find high-frequency adventitious shoot regeneration for similar genotypes, adequate concentrations, and a combination of growth regulators, such as auxins and cytokinin (Kinetin, zeatin, and thidizuron-N-Phenyl, N-1,2,3 thiadiazol-5 urea), should be controlled in this regard. Since the type of plant tissue and concentrations of plant growth regulators in plant cell culture can meaningfully affect the growth morphogenesis of plants [57] Liquid culture system is a critical step to enhance the multiplications rates of shoots produced *in vitro* [58]. The main properties of cytokinin include releasing lateral bud dormancy and stimulating cell division [59].

1.5 Vital components of medicinal plants

Plant growth regulators, including zeatin and thidiazuron (TDZ), and physical, chemical, and biological factors can affect the morphogenesis or organogenesis of plants. Shoot regeneration and development will vary among lingonberry clones [60]. Although most medicinal products are conventional, including those containing molecules derived from medicinal plants, in this case, isolated from the whole, contain a single PDMC as a chemical marker of reference. Cinnamon is an illustrative example of these two treatments: While the use of the cinnamon bark as an infusion to treat infectious diseases characterises the use of the medicinal plant; its primary, secondary

metabolite, cinnamaldehyde, which is isolated from the bark, proved to be an efficient antimicrobial agent [61] and can be used as a conventional medicament, similar to other types of synthetic antimicrobials.

*1.5.1 Chicory (*Cichicorium intybus L.*)*

Its species and use categorise Chicory; thus, industrial Chicory, also known as *C. intybus* L. var. sativum, is among the family of the Asteraceae, which is extensively used to produce inulin in South Africa, northern Europe, India, and Chile (Street et al., 2013). Although, the comprehension of the bioactive compounds that undergo synthesis by the biochemical pathways is farfetched [62]. Chicory as an essential plant contains four primary polyphenols: Caftaric, Chlorogenic, Isochlorogenic, and chicoric acids that are prominent in the type of *C. intybus* found in Nord-Pas-de-Calais, France [63]. Caffeic esters in Chicory have been portrayed to have antioxidant properties and potential therapeutic properties, such as anti-diabetic properties [64–66]. One of the essential phenolic compounds in Chicory is chicoric acid, also known as diacetyl tartaric acid; it is used to treat AIDS; thus, it serves as an anti-AIDS agent [67]. The root pulps of Chicory constitute a significant by-product of inulin producing industries and are used as feeds for animals. Extracts from chicory pulps contain a high quantity of pectin, a polysaccharide widely used as a gelling agent, stabiliser, and thickening agent in food [68]. Inulin is a soluble fibre that develops naturally in the chicory plant and has powerful medicinal benefits for human health, it controls and lowers fat, sugar, and calorie in the body, thus giving a tasty appeal, and it can be described as a natural fructan that tends to provide nutritional and health benefits when modified with oligo-fructose than when it is in a pure form [69].

1.5.2 S. rebaudiana Bertoni

S. rebaudiana is referred to as a medicinal plant owing to its natural sweet attribute. This natural resource is recommended for millions of diabetic patients as part of their daily intake since it serves as a natural sweetener and contains substances that promote wellness. Also, the leaves of Stevia comprise flavonoids, antioxidants, alkaloids, water-soluble chlorophylls, xanthophyll, water-soluble inert oligosaccharides, free sugars, amino acids, essential oils, trace elements, vitamins, hydroxycinnamic acids (Caffeic), and polyphenols. Thus, the low-calorie diterpenoid steviol glycosides found in the leaves of Stevia give a sweet taste that is almost 300 times sweeter than usual sucrose [70–72]. Steviol glycosides from the leaves of Stevia are rapidly being developed into an essential ingredient for the food industries for use as sweetener and flavour garnish. The biochemical constituents contained in the plant are of benefit to the pharmaceutical industry [73].

1.5.3 M. citriodora *(bee balm/*Cambridge scarlet*)*

Monarda plant contains an essential oil rich in phenolic monoterpenes, which differs according to its taxon and region of cultivation, and this was shown for medicinal plants. Thus, numerous species, such as *Monard didyma L.* (*M. citriode)* and *Monard fifistulosa* L., are used as medicinal, flavouring, and ornamental plants due to their composition. The hybrid crossing between these species may lead to dynamic hybrids with elevated decorative value and high essential oil contents [74–76]. The Monarda leaves and flowering stems contain water infusions that possess diuretic,

anti-helminthic, carminative, expectorant febrifuge, stimulant, and rubefacient properties, which help in the treatment of colds, headaches, and reduce insomnia, also acts as a stomach agent. The plant also possesses strong antifungal activity [77].

1.5.4 Rhodiola krylovii

Rhodiola krylovii is a medicinal plant with several compositions, such as polyphenols, which include; flavonoids, tyrosol, pro-anthocyanidins and cinnamyl alcohol glycosides, organic acids, essential oils, sugars, fats, alcohols, and proteins [78]. Several types of research have demonstrated that the main composition of the Rhodiola plant is tyrosol and salidroside. These compounds possess anticancer bioactivities, antifatigue, antidepressant, antioxidant, adapt genic, anti-inflammatory, and antinociceptive, modulate immune function and prevent cardiovascular, neuronal, liver, and skin disorders [79]. Due to their bioactivity, Rhodiola plant extracts, such as tyrosol and salidroside, tend to stop ageing and attenuate age-related diseases in humans and animals [80].

1.5.5 H. coronarium

H. coronarium (Sulla or French honeysuckle) is typified mainly by high percentages of Norisoprenoids as breakdown products of carotenoids, controlled by vomifoliol. Hence, the other main compounds include 3-hydroxy-4-phenylbutan-2-one and methyl syringate. These compounds are extractable natural volatiles and semi-volatiles distinct compounds, such as a small number of terpenes, norisoprenoids, benzene derivatives, aliphatic compounds, and Maillard reaction products, in the extracts signifies that *H. coronarium* is rather distinctive as compared to the other kinds of honey of the genus that gas chromatography and mass spectro photometer have chemically studied. However, specific markers of the honey botanical origin have not been discovered. In addition, the results obtained show that *H. coronarium* has the potential for a chemical characterisation since reliable data have been obtained so far by using high-performance liquid chromatography and headspace solid-phase micro-extraction in the initial study of *H. coronarium* natural volatiles that is extractable from Sardinian sulla honey samples. Including a variety of distinctive norisoprenoids, benzene derivatives, aliphatic compounds, and Maillard reaction products, but only a few terpenes were found [81, 82].

1.6 Uses and benefits of medicinal plants for medicine

Previous studies by researchers on medicinal plants have claimed that plants have been used for medicine about 60 thousand years ago, which paved the way for more discoveries of medicinal plants [83]. The use of medicinal plants for medication is progressively becoming a primary type of medication, mainly in tropical and underdeveloped countries. Medicinal plant utilisation has been adopted to prevent infectious diseases and avoid death in humans. Whereas, inadequate application of these plants may cause resistance to bacteria, resulting in the antibiotic crisis, along with the limited development of novel molecules [84].

The medicinal applications have been conducted with aid of some medicinal plants. For example, stevia extracts were used as intravenous infusions in rats to reveal their effect on glucose metabolism, diuresis, organ weights, and endocrine function anti-androgenic activity. Stevia extracts have shown some health benefits

since they serve as antioxidants and blood pressure and hypertension reducers [85]. The Monarda plant acts against pathogenic and microbial spoilage of food and promotes health. Examples of such organisms that this plant attacks include; Escherichia coli, Listeria monocytogenes, Staphylococcus aureus, and others Salmonella typhimurium or Pseudomonas aeruginosa [86, 87].

Medicinal leaf extracts have antibacterial and fungicidal properties, with an inhibitory effect on the growth of such bacteria as S. aureus, Streptococcus mutants, Bacillus subtilis, and E. coli [88]. Stevia's antihypertensive, anti-inflammatory, anticancer, antidiarrheal, diuretic, and immunomodulatory properties have been shown to this extent [70]. Rhodiola plant extracts are utilised traditionally in tonics and adaptogens and are incorporated in antidepressant and anti-inflammatory drugs [89, 90].

1.7 Factors influencing the cultivation of medicinal plants

Some factors influence the growth of plants (medicinal plants), which may affect many features and the quality of these plants. These factors include temperature, light, altitude, atmospheric humidity, soil, and rainfall:

a. Temperature: It is one of the significant factors that influence the cultivation of medicinal plants. An increase and decrease in temperature affect photosynthesis, and plant respiration rate is affected by causing an increase in respiration rate.

b. Light: It is an essential factor for medicinal plant cultivation growth. It aids the process of photosynthesis, germination process of seeds, flowering, vegetative growth, tuber formation, and the opening and closing of stomata in plants—the growth rate of plants increases during the night since the dark phase. Hence, High light intensity affects plant growth.

c. Altitude: It is a vital factor that influences the cultivation of medicinal plants. As a result of an increase in altitude, there is a decrease in temperature and atmospheric pressure. While the velocity of wind, relative humidity, and light intensity increase. Vegetative properties can change as a result of changes in altitude.

d. Atmospheric Humidity: It is an essential factor that can influence the transpiration rate in plants, thereby causing water evaporation from the earth's surface.

e. Soil/Nutrient Composition: Plants are grown on soil or in a substrate that contains vital nutrients. Thus, selected medicinal plant species species-specific medicinal plant parts can dictate optimal soil conditions, including soil type, drainage, moisture retention, fertility, and pH. The addition of excess fertiliser can accumulate soluble salts, which form a whitish crust on the growing medium's surface.

f. Water/Rainfall: In an external setting, rainfall influences plants' growth, such as the morphology and physiology of plants. For instance, continuous rainfall can cause water-soluble substances from the leaves and roots by leaching to be lost,

mainly associated with plants that produce glycoside and alkaloids [91]. High production and labour costs limit the commercial use of micropropagation [92].

1.8 Limitations of medicinal plant cultivation

There are shortcomings in medicinal plants' applications due to their exceptional qualities in the class of plants, and the products from these medicinal plants are essential sources for improving and maintaining human health [93]. Medicinal plant cultivation has been abortive due to the complexity of germination and environmental factors [38].

It can be clearly stated that the information about the use of medicinal plants is enormous, and in some circumstances, these natural resources are incorporated as the only form of preventing and curing diseases [94]. Anxiety around the introduction of medicinal plants in health systems is about how people utilise these products and how they will be recommended and presented to the population. Since specific consumers who may have some empirical knowledge about the use of certain medicinal plants may fall short of information concerning the actual toxicity of several species, a large number of the population still think that plants cannot damage their health. However, not all the users of these plants have this ideology about these natural resources.

What is more, is the fact that medicinal plants have a variety of species that are known by the same name and when misused, can lead to allergy and stimulation of medical conditions. There is inadequate knowledge of how the preparation and contraindications of the plants, lack of knowledge by health professionals regarding the side effects after consumption, difficulty regarding correct usage of these natural resources, and unreliable information about these plants, which leads to difficulty disseminating this to people. Complementary practices in administering these natural resources to patients to treat specific ailments are abandoned [95].

The storability of medicinal plants plays a role in the ineffective use of the products since many biochemical differences can occur within the natural resources leading to changes in bioactivity [96].

2. Materials and methods

2.1 Experimental location

Experiments were conducted in the Department of Technology for Organic Synthesis, Institute of Chemical Engineering Ural Federal University, Ekaterinburg, Russia.

2.2 Raw material location

The raw materials included *C. intybus, S. rebaudiana Bertoni, Monarda citriodora, Rhodiola krylovii Chicory, Stevia, Cambridge scarlet (M. didyma), Rhodiola krylovii* and were all obtained within Russian Federation.

2.3 Raw material consideration

The primary raw material consideration for the *in vitro* cultivation of these plant cells includes solid and liquid culture media with enough nutrient composition required for the plant cells' growth.

2.3.1 Materials/equipment for experiment

2.3.1.1 Reagents for media

Sucrose, solution of iron chelate, macronutrients, micronutrients, vitamins, Kinetin, 1-naphthalene acetic acid (NAA), dichlorophenoxyacetic acid, agar, and distilled water 100 ml.

2.3.2 Reagents for plant cells sterilisation

Ethanol (70%), sodium hypochlorite (3%), Tween detergent, sterile water, carboxylic acid amide (CAA) fungicide (2%).

2.3.3 Equipment

Climate chamber, Petri dishes, laminar system, test tubes, autoclave, oven, stirrer, laboratory glassware (conical flask, beakers, burette), micropipette and pipette tips, knife, forceps, filter paper, foil paper, electric stove, refrigerator, pincers, Bunsen burner, thermometer, and pH meter.

2.3.4 Methods

Different methods/procedures were used to cultivate *C. intybus, S. rebaudiana Bertoni, Monarda citriodora and Rhodiola krylovii Stevia, Chicory Cambridge scarlet, and Rhodiola krylovii* to identify which sterilisation method was best for each plant type and which procedure would not incur contamination. Studying the effectiveness of various sterilisation methods for seed cultivation on solid media in tubes for callus formation (medium M&S + kinetin) and cultivating seeds on solid media in tubes to obtain sterile micro plants (medium M&S) were carried out. The germination of non-sterile/sterile seeds and obtaining non-sterile and sterile plant seedlings in the Petri dish were carried out. Growing sterile micro explants for callus formation (medium M&S + kinetin) and preparation of cell suspension cultures for wet and dry weight determination.

2.3.5 Media formulation

The media for this research was formulated from Murashige and Skoog Culture Media (1962). The Murashige and Skoog Medium is a shared medium used to cultivate laboratory plant cells. The discovery was made by two Plant scientists, Toshio Murashige and Folke K. Skoog [97], searching for a new type of plant growth regulator. Using Murashige and Skoog medium supplemented with 3 g of sucrose, 0.5 ml solution of iron chelates, 5 ml of macronutrients, 0.1 ml of micronutrients, 0.1 ml of vitamins, 0. ml of Kinetin, 0.25 ml of 1-naphthalene acetic acid (NAA), 0.8 to 0.9 ml of agar, and 100 ml distilled water for every volume of 100 ml media.

2.3.6 Preparation of solid culture media using Murashige and Skoog method

A total of 100 ml of distilled water was poured into a 250 ml round bottom flask. A weighing balance was used to measure 0.8 g of agar added to the water. The sample was placed on a heating device (infrared cooker) at the temperature of 60°C; 0.25 ml of 1-naphthalene acetic acid (NAA) was measured with a micropipette and poured

into the sample, and stirred. Then, 3 g of sucrose was added to the sample on heating and stirred, followed by adding 0.1 ml of Kinetin and 0.5 ml of chelate were added to the heating sample. After 10 mins, the sample was removed from the heating device and allowed to cool at room temperature (25°C). Then, 5 ml of macronutrients, 0.1 ml of micronutrients, and 0.1 ml of vitamin were added to the sample as the temperature reached 40°C. The sample was stirred and covered with cotton and foil paper with a plastic band around the cover and kept in the refrigerator to solidify for over 12 hours. The media was sterilised in an autoclave for 1 hour at 121°C. The sample was placed on a heating device for 20 mins at 60°C, and after heating, the sample was left to cool for 10 mins before transferring to test tubes.

2.3.7 Preparation of liquid culture media using Murashige and Skoog method

A total of 100 ml of distilled water was added to a 250 ml round bottom flask. The sample was placed on a heating device (infrared cooker) at a temperature of 60 °C and 0.125 ml of 1-naphthalene acetic acid (NAA) and 0.1 ml of dichlorophenoxyacetic acid, whereas measured with a micropipette and poured into the sample and stirred. Next, 3 g of sucrose was added to the sample while heating and stirring.

Then, 0.1 ml of Kinetin and 0.5 ml of chelate were added to the heating sample. After 10 mins, the sample was removed from the heating device and allowed to cool at room temperature before adding 5 ml of macronutrients and 0.1 ml of micronutrients. A total of 0.1 ml of the vitamin was added to the sample when the temperature was 40° C. The sample was stirred and covered with cotton and foil paper with a plastic band around the cover before it was preserved and kept in the refrigerator to solidify for more than 12 hours. The media was sterilised in an autoclave for 1 hour at 121°C. The sample was placed on a heating device for 20 mins at 60°C, and the sample was left to cool for 10 mins before transferring into a sterile flask. Different for callus cultivation.

2.3.8 Experimental design

2.3.8.1 Seed sterilisation

Studying the effectiveness of various sterilisation methods (A, B, C, and D) for seed cultivation on solid media in tubes for callus formation (medium M&S + kinetin) and the cultivation of seeds on solid media in tubes to obtain sterile micro plants (medium M&S).

I. Using 0.8 grams of agar in the solid media (the amount of agar used for inoculation of cells was 0.8 grams).

1. **Method A – Sterilisation and Cultivation Process of Stevia, Chicory, Bee Balm (Cambridge Scarlet), and Rhodiola krylovii Cells.**

   The volume of 400 ml of media with Kinetin was prepared using the above reagents for plant cells. However, 7 ml of solid media was poured into 52 test tubes. Then, sterile Petri dishes were used for the sterilisation process of seeds and seeds were first sterilised with NaOCl for 3 mins. Then, the seeds were transferred into Petri dishes containing ethanol for 1 min. The seeds were rinsed with sterile water for 2 mins. The seeds were left to dry before transferring them into the test tubes. Two seeds of Stevia, Chicory,

Bee Balm (Cambridge Scarlet), and Rhodiola krylovii cells were put into 13 labelled separate test tubes for each type of plant and were labelled. The test tubes containing samples were incubated with a light intensity of 16 hours and dark phase of 8 hours, a temperature of 250C (±10C) and relative humidity of 50%. After 14 days, a percentage of contamination was carried out.

2. **Method B – Sterilisation and Cultivation process of Stevia, Chicory, Bee Balm (Cambridge Scarlet), and Rhodiola krylovii Cells.**

   The 600 ml of M and S media with Kinetin was prepared using the above 11 reagents for plant cells. A total of 7 ml of solid media was poured into 80 12 test tubes. At the same time, sterile Psetri dishes were used to sterilise seeds. Seeds were sterilised using 70% ethanol for 2 mins. The rinsing of seeds was done with sterile water for 1 min. Seeds were washed with NaOCl for 1 min. Then, rinsing of seeds with sterile water for 1 min. The seeds were allowed to dry for a few mins. Two seeds were put in each test tube (20) for each type of plant and well labelled. The samples were placed in the climate chamber for incubation. Then, the test tubes containing samples were placed in the climate chamber and incubated under conditions for incubation with 16 hours of light phase and 8 hours of no light phase and temperature 250C (± 1°C) and relative humidity of 50%. After 14 days, the percentage of contamination was determined.

II. Using 0.9 g of agar in the solid media (the amount of agar was increased from 0.8 grams to 0.9 grams to reach average thickness).

1. **Method C – Sterilisation and Cultivation Process of Stevia, Chicory, Bee Balm (Cambridge Scarlet), and Rhodiola krylovii Cells.**

   The 400 ml of M and S media with Kinetin was prepared using the above reagents for plant cell culture. A total of 9 ml of media was poured into 40 sterilised test tubes. Sterile Petri dishes were used for the sterilisation process of seeds. Seeds were thoroughly washed in flowing water. Afterwards, seeds were sterilised with 2% carboxylic acid amide (CAA) fungicide for 2 mins, followed by washing seeds with 70% ethanol for 2 mins, then 3% NaOCl solution for 2 mins. Rinsing seeds thoroughly with sterile water thrice (three times) in three separate sterilised Petri dishes for 5 mins. Two seeds of Chicory and Stevia were put into 10 different test tubes containing solid media, respectively. Then, samples were placed in the climate chamber for incubation with no light intensity, temperature 250C (± 1 °C), and relative humidity of 50%. After 14 days, the percentage of contamination was determined.

2. **Method D – Sterilisation and Cultivation Process of Stevia, Chicory, Bee Balm (Cambridge Scarlet), and Rhodiola krylovii Cells.**

   The 400 ml of M and S media volume with Kinetin was prepared using the above reagents for plant cells. A total of 9 ml of solid media was poured into 56 test tubes. Sterile Petri dishes were used for the sterilisation process

of seeds. Seeds were washed adequately with an antibacterial soap in flowing water for 10 mins. Seeds were sterilised with 70% ethanol for 2 mins. Seeds were properly washed three times with sterile water. Then, the seeds were sterilised with 3% NaOCl solution for 5 mins. Seeds were later transferred into sterile test tubes containing solid media. Fourteen labelled, sterile test tubes contained Stevia, Chicory, Bee Balm (Cambridge Scarlet), and *Rhodiola krylovii* cells. Seeds were kept in the climate chamber for incubation with no light intensity, temperature 250C (± 1 ° C), and relative humidity of 50%. After 14 days, the percentage of contamination was determined.

The level of percentage contamination for the four sterilisation methods (A, B, C, and D) for seed cultivation on solid media in tubes for callus formation (medium M&S + kinetin) and the cultivation of seeds on solid media in tubes to obtain sterile micro plants (medium M&S) was determined by using the formula:

$$\% \text{ of Contamination} = (\text{Number of Contaminated Test tubes with cells})/(\text{Total Number of Test tubes with seeds}) * 100 \quad (1)$$

2.3.8.2 The germination of non-sterile/sterile seeds and obtaining non-sterile and sterile plant seedlings in Petri dishes

A. Non-sterile Plant Seedlings

The seeds of Cambridge scarlet, Rhodiola krylovii, and Chicory were put into six Petri dishes. Two Petri dishes for Bee Balm (Cambridge scarlet), two for Rhodiola krylovii, and two Petri dishes for Chicory were prepared. The seeds were unwashed, and 7 ml of distilled water was added to all Petri dishes. The samples were labelled and sealed with paraffin and incubated for 2 weeks for plant growth in a climate chamber for a plant with no light intensity, a temperature of 250C (±1°C), and relative humidity of 50%.

B. Sterile Plant Seedlings

A total of 7 ml of sterile water was poured into six sterile Petri dishes. Seeds of Chicory, Rhodiola krylovii, and Cambridge scarlet were disinfected with 70% ethanol for 2 mins and 3% NaOCl solution for 2 mins. Seeds were rinsed thoroughly with sterile water twice in separate sterilised Petri dishes for 2 mins. The seeds were poured into the six sterilised Petri dishes and sealed with paraffin. Samples were labelled and incubated in the climate chamber for 2 weeks with no light intensity, temperature 25°C (1°C), and relative humidity of 50%.

2.3.8.3 Growing sterile explants for callus formation (Medium M&S + kinetin)

I. Growth Media with Kinetin (+Kinetin)

The media for growing callus was slightly different, with a variance in supplements (Murashige and Skoog media, 1962). A 300 ml M and S media volume was prepared, and 9 ml was poured into 24 sterile test tubes. The non-

sterile seedlings from the six Petri dishes containing Chicory, Rhodiola krylovii, and Cambridge scarlet were transferred to the test tubes after cutting and disinfection: First roots of seedlings were cut, and explants were disinfected using 2% carboxylic acid amide (CAA) fungicide for 2 mins, followed by the washing of explants in 70% ethanol for 2 mins. Explants were rinsed with sterile water. Seeds were washed with 3% NaOCl for 2 mins and rinsed with sterile water. Explants were transferred into labelled sterile test tubes and were incubated for 2 weeks with conditions of no light intensity, a temperature of 250C (±1 °C), and relative humidity of 50%.

II. Growth Media without Kinetin (-Kinetin)

The volume of 600 ml of M and S media without Kinetin was prepared using the above reagents for plant cells. Then, 9 ml of media was poured into 60 sterilised test tubes each. Sterile seedlings from the six Petri dishes containing Chicory, Bee balm (Cambridge scarlet), and Rhodiola krylovii were cut and thoroughly washed. Disinfection of explants was done using 2% carboxylic acid amide (CAA) fungicide for 2 mins followed by washing with 70% ethanol for 2 mins and then with 3% NaOCl solution for 2mins. Rinsing of explants thoroughly with sterile water thrice (three times) in three separate sterilised Petri dishes for 5 mins was carefully carried out. The three plants' explants were put into 20 labelled sterilised test tubes, respectively, and the remaining media was preserved in the refrigerator. Incubation of explants was done under conditions of no light intensity, the temperature of 25°C (1°C), and relative humidity of 50% for a period of 2 weeks.@

The formula below was used to determine the percentage contamination for the above experiments:

$$\%\ \text{of Contamination} = (\text{Number of Contaminated Test tubes with cells})/(\text{Total Number of Test tubes with cells}) * 100 \quad (2)$$

2.3.8.4 Preparation of cell suspension cultures

The callus of Chicory was transferred into two sterile flasks containing M and S cell suspension cultures of Kinetin and without Kinetin. Samples were crushed with a knife and stirred vigorously to aid the formation of more cells. The samples were incubated in plant-controlled equipment under conditions of continued shaking with 16 hours of light intensity phase at 24(±1)C and 8 hours of dark phase at 18 (±1) 0C and after 1 week, the callus was transferred to a new cell suspension culture maintaining the conditions for a week. One week later, the callus in both flasks was transferred to six sterile flasks using the same media formulation; three flasks had no kinetin, while three had Kinetin in the media. A total of 10 ml of starter culture (cell suspension) and 50 ml of liquid media were poured into the six labelled sterile flasks, and the samples were returned to the plant-controlled equipment to grow to maintain the conditions of continuous shaking with 24 hours of light at 24 °C (±1) for 4 weeks. Each week the samples were checked to ensure that they were free from contamination.

2.4 Microscopy

The overall magnification can be calculated as the product of the lenses and the distance over which the image is projected:

$$M = (D * M1 * M2)/250 \text{ mm} \quad (3)$$

where D = projection (tube) length (usually = 250 mm); M1, M2 = magnification of objective and ocular. Thus, 250 mm = minimum distance of distinct vision for 20/20 eyes [98]. Callus of Chicory grown using cell suspension culture (liquid media) of + Kinetin and – Kinetin was viewed under the microscope using a 10 mm focal point.

2.5 Wet and dry weight analysis

Drying process was achieved by initially setting the oven temperature at 60°C. 5 ml of Kinetin containing media starter was pipetted into three separate centrifuge tubes and properly labelled. Afterwards, 5 ml of media without Kinetin was pipetted into three different and well labelled centrifuge tubes. Centrifugation was done for 10 mins on 5000 rpm at 15°C; thus, the aliquots were separated, and 5 ml of sterile water was added to the supernatants and centrifugation was done twice. Six filter papers (2.5 cm) were dried in the oven for 9 hours at 60°C. Then, the empty weight of filter papers was obtained, followed by the weight of filter papers with samples. The samples were later placed in the oven for 24 hours at 60°C. The weight of the dried samples on the filter papers were obtained. The fresh and dry weights were determined one week after transferring the seeds into a new culture media.

$$\text{Wet wt (g/L)} = (\text{Wt of wet filter paper} + \text{cells (g) wt of wet filter paper(g)})/ (\text{Sample vol (mL)}) \text{ x } 100 \quad (4)$$

$$\text{Dry wt (g/L)} = \left(\text{Wt of dry filter paper} + \text{cells (g) wt of dry filter paper(g)}\right)/ (\text{Sample vol (mL)}) \text{ x } 100 \quad (5)$$

2.6 Charts

Bar charts and area charts were used to present the data obtained from the experiments.

3. Results and discussion

3.1 Seed sterilisation: Studying the effectiveness of various sterilisation methods

Results were obtained for the four sterilisation methods after 14 days of cultivating seeds of Stevia, Bee balm, Chicory, and *Rhodiola krylovii*, and the values were presented using tables and bar charts (see **Table 2**).

3.1.1 Method a

The result of method A as shown in **Figure 1**, Stevia cells had 53.9% contamination, which was the least contaminated. While Bee balm, Chicory, and *Rhodiola krylovii* had

PLANTS	METHOD A			METHOD B			METHOD C			METHOD D		
	Total No. of Test tubes with micro plant s	No. of Contaminated. Test tubes	No. Non-contaminated. test tubes with micro plant s	Total No. of Test tubes with micro plant s	No. of Contaminated. Test tubes	No. Non-contaminated. test tubes with micro plant s	Total No. of Test tubes with micro plant s	No. of Contaminated. Test tubes	No. Non-contaminated. test tubes with micro plant s	Total No. of Test tubes with micro plants	No. of Contaminated. Test tubes	No. Non-contaminated. test tubes with micro plant s
Stevia	13	7	6	20	11	9	10	5	5	14	2	12
Bee Balm	13	13	0	20	20	0	10	10	0	14	14	0
Chicory	13	12	1	20	18	1	10	3	7	14	7	7
Rhodiola	13	13	0	20	20	0	10	10	0	14	14	0

Table 2.
Effectiveness of various sterilisation methods (A, B, C and D).

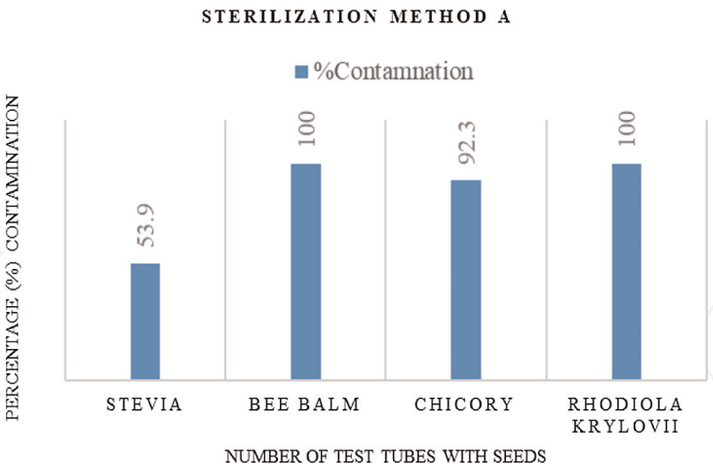


Figure 1.
Chart showing percentage contamination of method A.

100%, 92.3%, and 100%, 92.3%, and 100% contamination, respectively. The result shows that this method is averagely suitable for Stevia seeds but not suitable for Bee balm, Chicory, and Rhodiola krylovii seeds. The sterilisation method is not effective for all the seeds since the level of percentage contamination is high, and the agar composition in this 05 method was 0.8 g.

3.1.2 Method B

The result of sterilisation in Method B **Figure 2** shows that Chicory had 40% contamination and Stevia had 55% contamination. In contrast, Bee balm and *Rhodiola krylovii* had a 100% level of contamination. This sterilisation method of plant seeds is suitable for Chicory (40%) since the contamination percentage was few. Stevia seeds also can be sterilised using this method because the percentage level of contamination after cultivation was 55%. Nevertheless, this method is unsuitable for Bee balm and *Rhodiola krylovii* since the level was very high (100%), as shown in **Figure 2** and **Table 3**, and the agar composition in this method was 0.8 g, and this could have affected the growth rate of the plants.

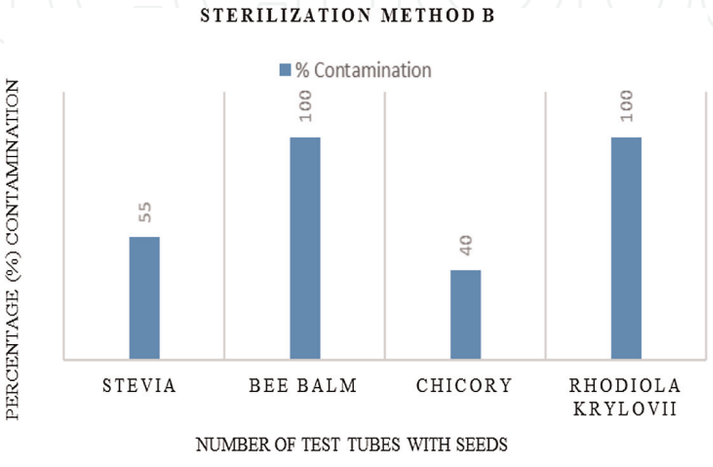


Figure 2.
Chart showing percentage contamination of method B.

PLANTS	Culture Media with Kinetin (+Kinetin)			Culture Media without Kinetin (-Kinetin)		
	Total Number of Test tubes with cells	Number of Contaminated Test tubes	Number of Non-contaminated Test tubes	Total Number of Test tubes with cells	Number of Contaminated Test tubes	Number of Non-contaminated Test tubes
Chicory	8	5	3	20	20	0
Bee Balm	8	6	2	20	20	0
Rhodiola krylovii	8	5	3	20	20	0

Table 3.
Growing sterile explants for callus formation (Solid Medium M&S + kinetin and – Kinetin).

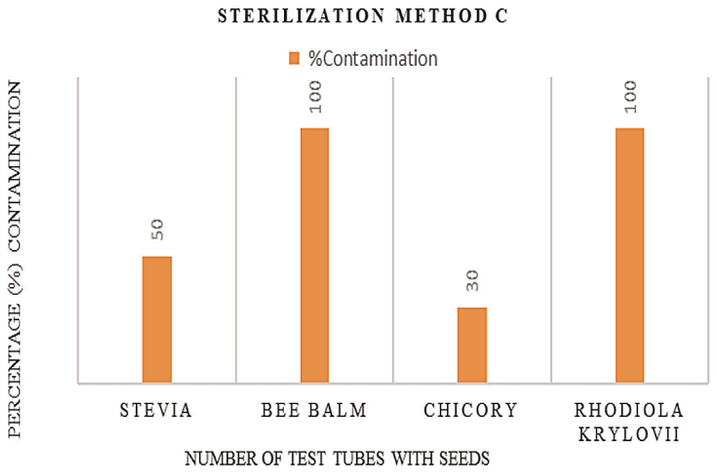


Figure 3.
The chart showing percentage contamination of method C comes after the subheading method C.

3.1.3 Method C

The percentage (%) contamination was determined for method C, and from the result, as shown in **Figure 3**, Chicory and Stevia had 30% and 50% contamination, respectively, while Bee balm and *Rhodiola krylovii* both had 100% contamination. Method C is a suitable sterilisation method for Chicory seeds. The agar composition used for media preparation in this method was 0.9 g, which could have influenced the plant seeds.

3.1.4 Method D

Figure 4 shows the level of percentage (%) of Stevia (14.29), Bee balm (100%), Chicory (50%), and Rhodiola krylovii (100%). The result obtained using method D indicates that Stevia seeds had a minor percentage (%) contamination, which is 14.29%, and it is the best method for sterilising stevia cells. Although Chicory seeds can also be sterilised using this method, it stands an average chance of not getting contaminated than Stevia, which has a greater chance of no contamination. While Bee balm and Rhodiola krylovii had 100% contamination, thus this method of sterilisation is not suitable for both plant seeds, and the agar composition used during the media preparation was 0.9 g.

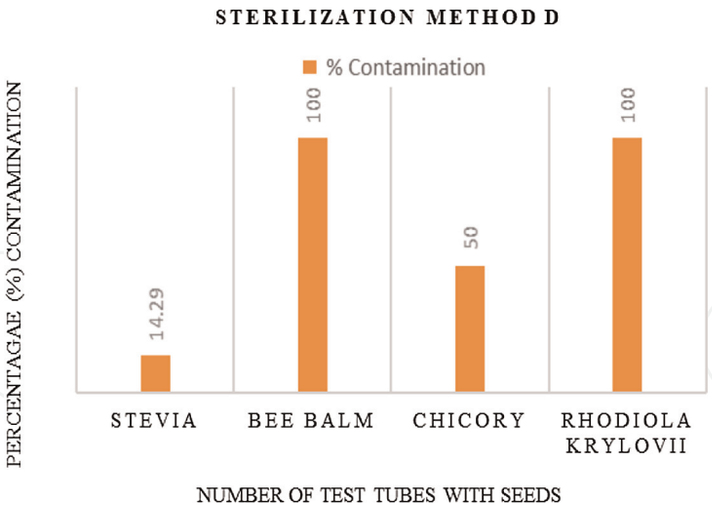


Figure 4.
Chart showing percentage contamination of method D.

From the four (4) methods of sterilisation used in this experiment, the result for each Method (A, B, C, and D) Stevia and Chicory cells were less prone to 100% contamination when compared to Bee balm and Rhodiola krylovii, which turned out to have 100% contamination in the four methods of sterilisation and cultivation. The most suitable method for Stevia is method D which only had 14.29% contamination, and this result can be linked to the findings of Halim et al., 2016 for the sterilisation process of Stevia, which had 15% contaminated plant cell culture. The best method for cultivating Chicory cells was method C, which had a 30% (%) contamination level. Hence, it can be stated that different plant cells have a specific method of sterilisation that enables cells/seeds to be free from pathogenic microorganisms that may affect the cultivation process. However, the composition of culture media might have influenced the growth rate of the plants, and this agrees with the findings of Yildiz and Usha et al. [57, 99] that the higher the composition of plant regulators used, the higher the observable differences and the amount of agar used can affect the growth of plant cells. A total of 0.9 g of agar was used in methods C and D. In comparison, 0.8 g was used for methods A and B. Although, other factors, such as percentage concentration, application period, and temperature of NaOCl could, affect *in vitro* germination of seeds, regeneration potential of explants, and growth of seedlings when these factors are not taken into consideration. Hence, these results might also lead to seed contamination due to the inefficacy of NaOCl used during seed disinfection [100] (see **Figures 5–8**).

3.2 Germination of non-sterile/sterile seeds and obtaining non-sterile and sterile plant seedlings in Petri dishes

Two factors were considered under this experiment; a) non-sterile plant seedlings and b) sterile plant seedlings, and the plants were observed after 2 weeks of culturing. The observable differences between the plant cells of Chicory, Bee balm, and *Rhodiola krylovii* were determined by sight. The variation of growth between the plant cells was evident to the eyes. This experiment was done to obtain callus. Non-sterile Chicory showed moderate growth in the Petri dishes, while the growth of the pure Chicory was prominent. The non-sterile Bee balm showed no growth in the petri dish, unlike the sterile Bee balm was showed noticeable growth (sprouts). Non-sterile *Rhodiola krylovii* showed noticeable sprouts, while sterile *Rhodiola krylovii* showed noticeable sprouting.

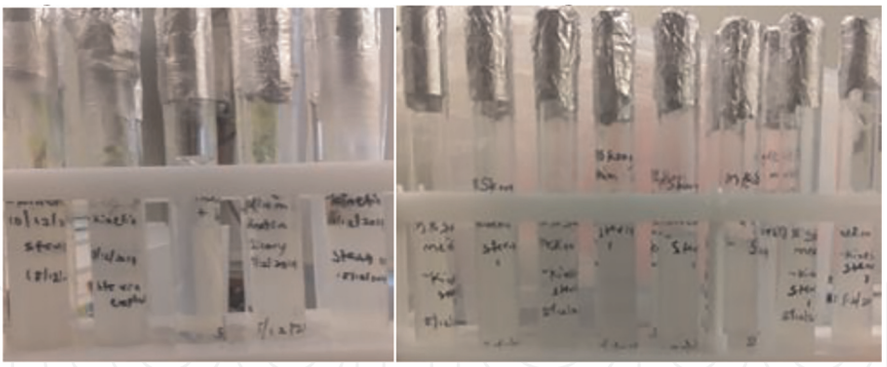


Figure 5.
Cultured stevia seeds in solid media.

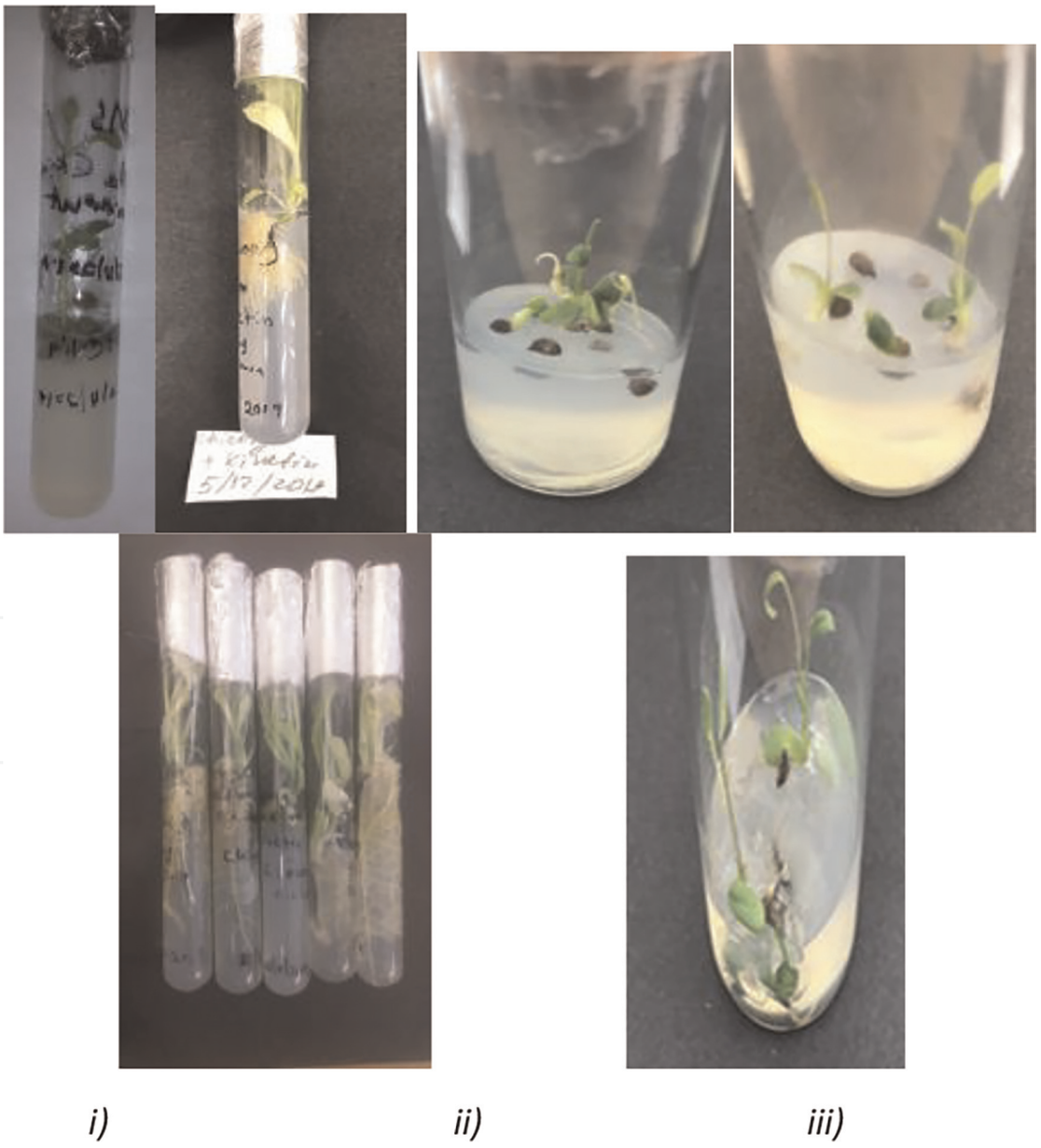


Figure 6.
(i) Stevia, (ii) chicory, and (iii) Hedysarum.

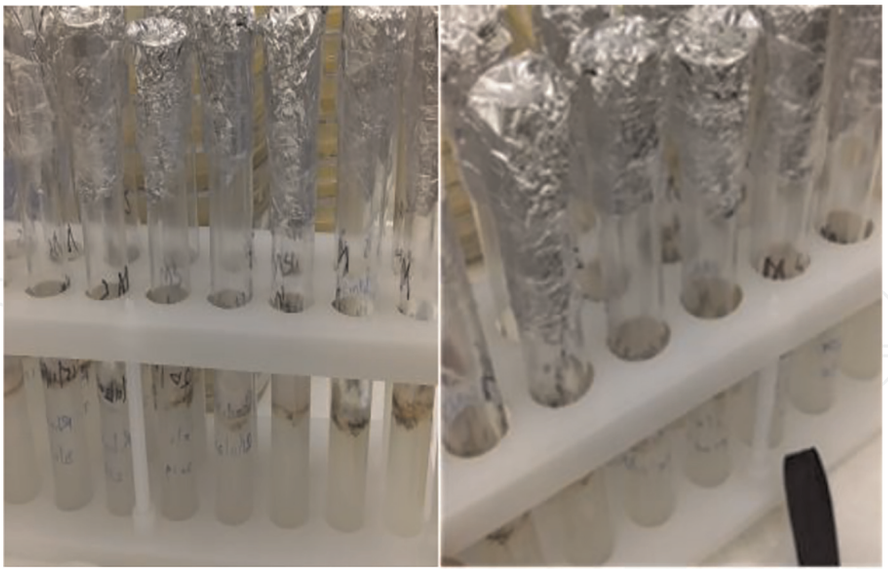


Figure 7.
*Contaminated samples of bee balm (*Cambridge scarlet*) and* Rhodiola krylovii *plants.*

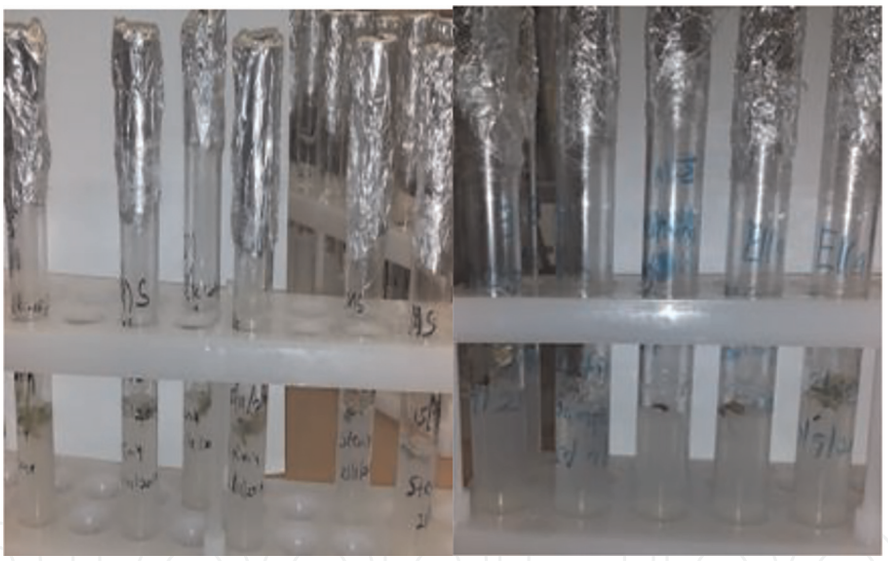
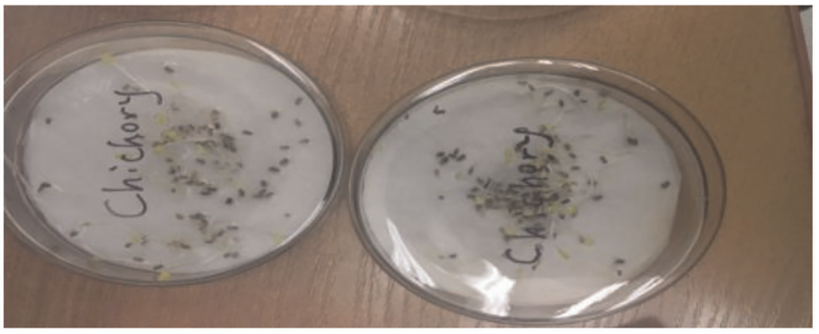


Figure 8.
(i) Growing stage of stevia and (ii) growing stage of chicory.

Hence, this indicates that sterilisation of plant cells is essential in the *in vitro* process of culturing seeds, and basic steps must be considered during sterilisation. The difference in growth between the sterile and non-sterile plant cells might result from their genetic differences among the two subspecies of the used plants. This result agrees with the findings of Debnath and McRae [60], which explained that different genotypes variably react for shoot proliferation and regeneration in a liquid medium or a semi-solid media in his work on a semi-solid medium between subspecies. Also, the water absorption rate can influence the seedlings' rate during *in vitro* cultivation of plant cells [101].

3.2.1 Non-sterile seedlings

The seeds were not sterilised (see **Figure 9**).



(i)

(ii)

(iii)

Figure 9.
(i) Non-sterile Chicory with sprouts after 2-weeks. (ii) Non-sterile Cambridge scarlet without sprouts after 2-weeks. (iii) Non-sterile Rhodiola krylovii with sprouts after 2-weeks.

3.2.2 Sterile plant seedlings (seeds were sterilised)

No Contamination after 2 weeks (see **Figure 10**).

3.3 Growing sterile explants for callus formation (Solid Medium M&S + kinetin and - kinetin)

Two factors were considered during the cultivation process of Callus of Chicory, Rhodiola krylovii, and Beebalm. These factors were (I) Growth Media with Kinetin

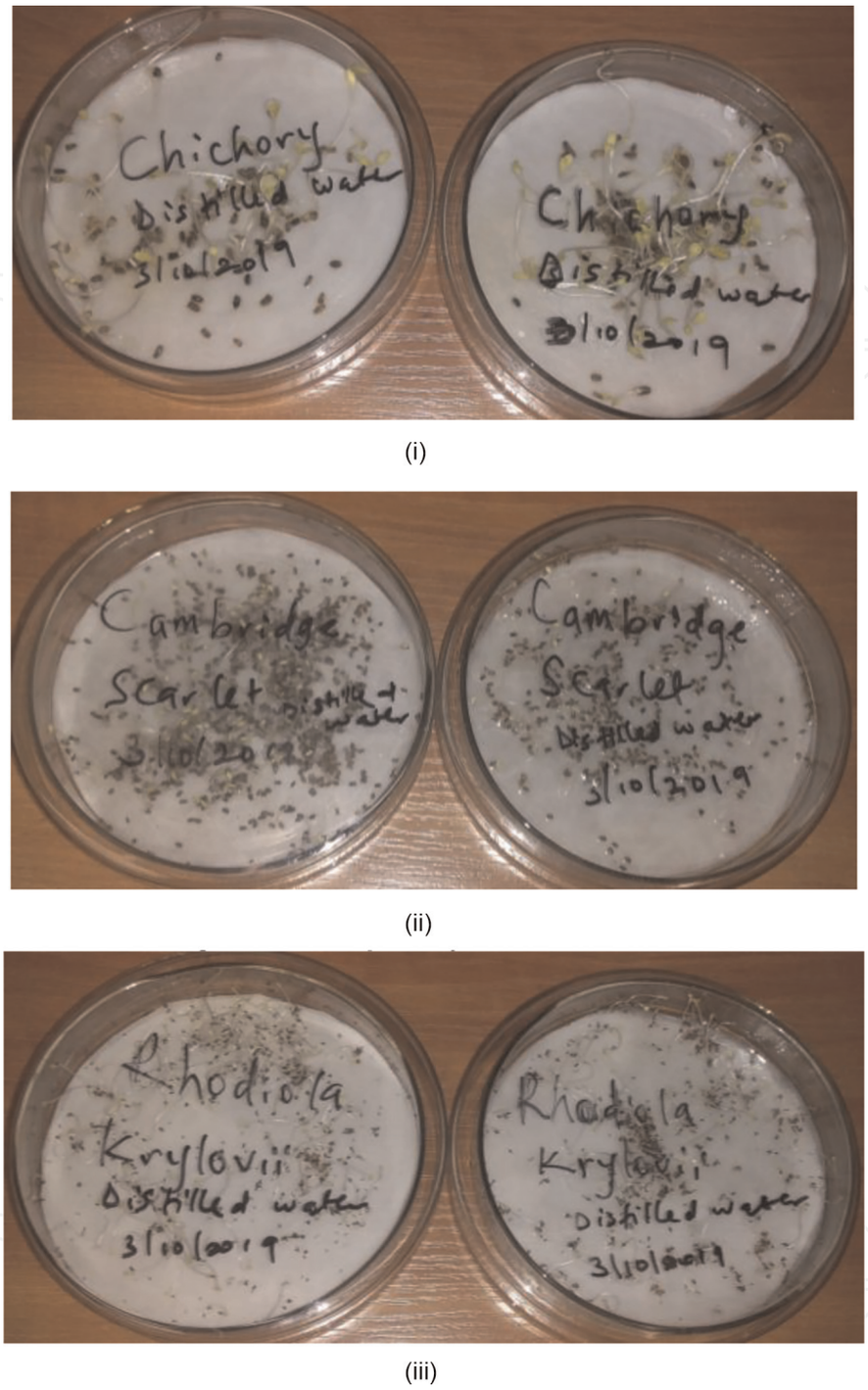


Figure 10.
(i) Sterile Chicory with sprouts after 2-weeks. (ii) Sterile Cambridge scarlet with sprouts after 2-weeks. (iii) Sterile Rhodiola krylovii with sprouts after 2-weeks.

(+ Kinetin) and (II) Growth Media without Kinetin (-Kinetin) and the determination of percentage (%)contamination of callus in test tubes after 2 weeks was done to know the percentage contamination of each plants using different media.

Growth of callus was observed with samples in media containing Kinetin. However, there was 62.5–75% contamination in the media containing Kinetin, as shown in **Figure 11**. Chicory and Rhodiola krylovii had the same level of percentage contamination, which was 62.5% and less than the percentage contamination of Bee balm.

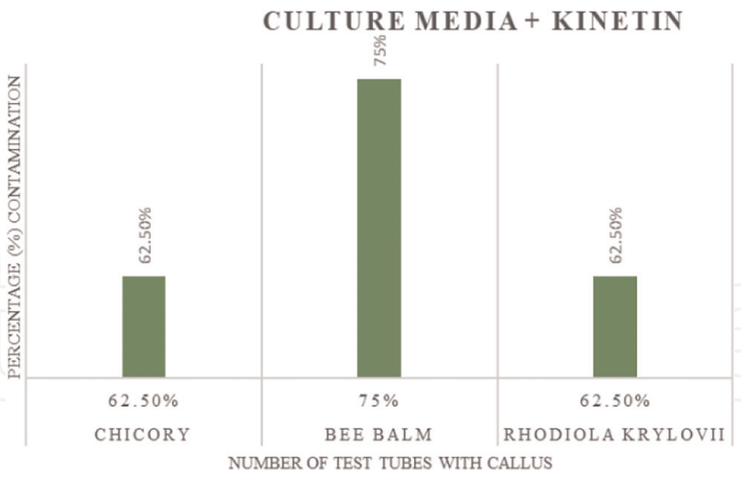


Figure 11.
Percentage contamination for callus formation (Medium M&S + kinetin).

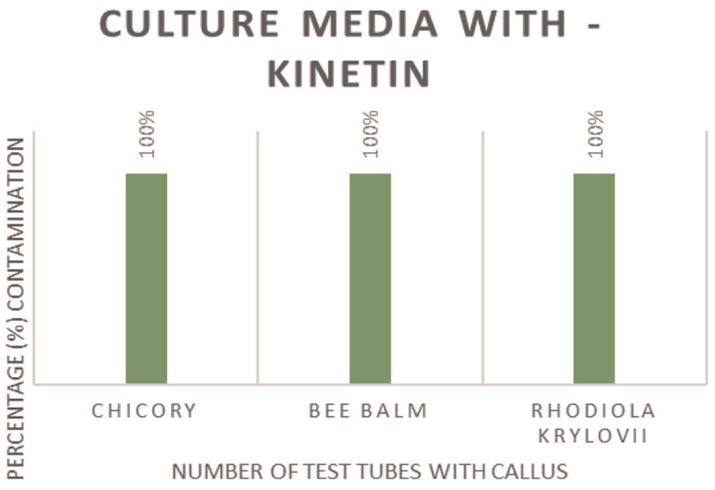


Figure 12.
Percentage contamination for callus formation (Medium M&S - kinetin).

After 2-weeks, no sign of growth was observed from any of the samples in the media with no Kinetin and all samples were contaminated. As shown in **Figure 12** of growth media without Kinetin, the level of contamination was 100% for all plants, and no growth was observed in all plant cultures. Thus, this can be due to the absence of Kinetin, a plant hormone that helps induce callus and shoot regeneration formation. While the high level of contamination can be a result of the poor method of sterilisation and the efficacy of sodium hypochlorite, this agrees with the studies of Racoppi [102], which reported that the sterilising agent NaOCl (Sodium Hypochlorite) could decrease in its activity when all the parameters, such as temperature and pH, are not stable. However, if the temperature is slightly above 10 °C, this might increase disinfection activity since this enables easy incursion into the seed coat. Higher temperatures of NaOCl affected the morphology of seedlings, resulting in abnormal growth of hypocotyls and other parts of the plant cells. The unsuccessful result of no growth must have been due to variation in room temperature. According to Yildiz [57], change in temperature of (±1°C) can have an adverse effect on the plant, resulting in no growth (see **Figures 13–15**).

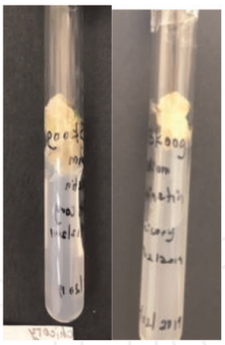


Figure 13.
Callus of chicory in solid culture media after 8 weeks.

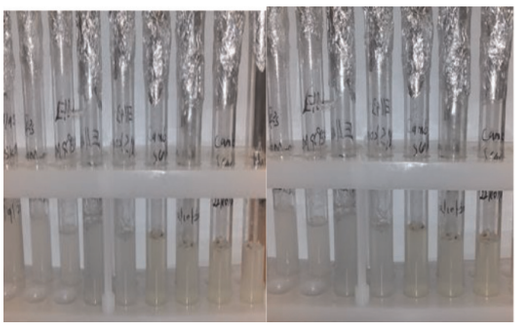


Figure 14.
*Bee balm (*Cambridge scarlet*) and* Rhodiola krylovii *in solid media with no growth of callus.*

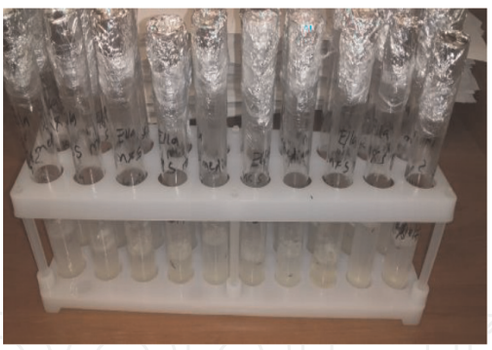


Figure 15.
*Stevia, chicory and bee balm (*Cambridge scarlet*),* Rhodiola krylovii *in solid media showing no growth sign after 4 weeks of culture.*

3.4 Preparation of cell suspension cultures

Two factors were used to determine the growth of callus in cell suspension (liquid media), and these are the use of the growth regulator kinetin and the absence of growth regulator – Kinetin in the media with the aid of microscopy results were obtained (see **Figures 16** and **17**).

3.5 Microscopy results of callus of chicory

a. The cells were viewed under the microscope after 1 week of culturing the callus

See **Figure 18**.

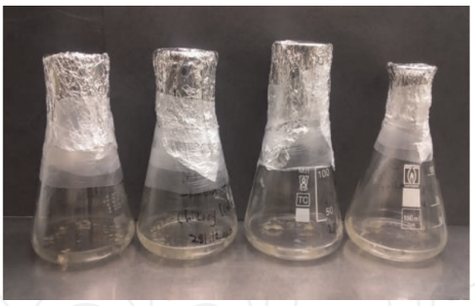


Figure 16.
Callus of chicory in liquid media of + kinetin and – Kinetin.

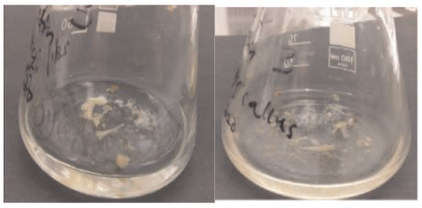


Figure 17.
Callus of chicory in the cell suspension.

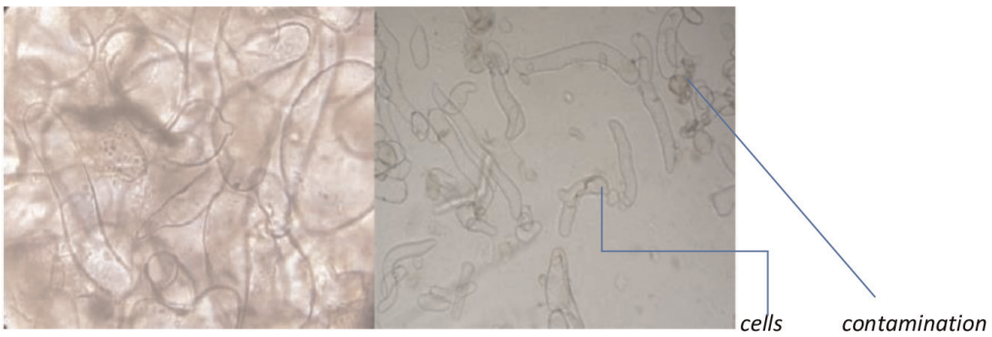


Figure 18.
(i) Chicory cells in liquid media (+kinetin). (ii) chicory cells in liquid media (− kinetin).

b. Cells after 2 weeks of Transferring into new Liquid culture media

See **Figure 19**.

The microscopy result after culturing the callus in an M and S liquid media of (−) and (+) Kinetin shows that the cells from the media with Kinetin developed more giant cells and several cells after 1 week of cultivation, while the cells in the media with Kinetin developed smaller and multiple cells. However, after 4-weeks, the cells from the media with Kinetin developed more giant cells in more significant numbers. In contrast, the cells from the media without Kinetin incurred contamination before the fourth week, and this result agrees with the findings of Usha et al. and Debnath [99, 103], which explains the necessity of administering a 14-day dark treatment for callus formation and the shoot regeneration of plants. The variation between the cells in media with Kinetin and media without Kinetin could be due to the addition of Kinetin, a growth regulator. It regulates the growth processes of cells by controlling cell division and cell differentiation affecting the plant's formation and regeneration process. Also, this correlates with the results of Usha et al. [99] that envaulted the

DOI: http://dx.doi.org/10.5772/intechopen.104650

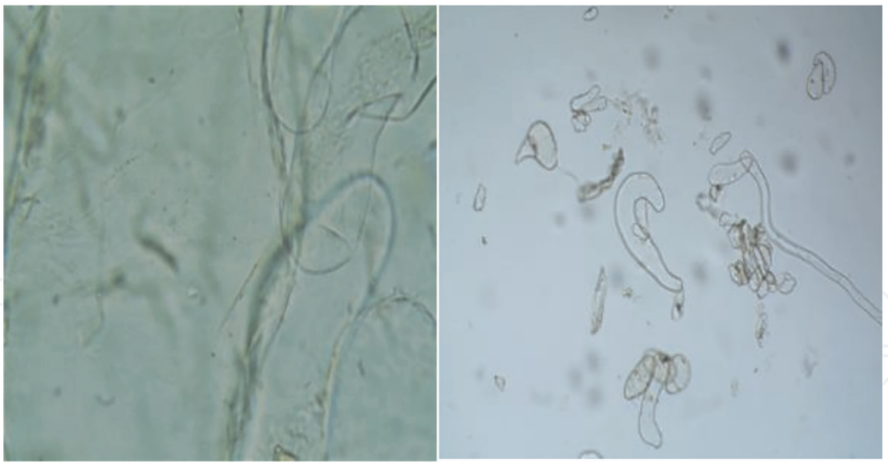


Figure 19.
(i) Chicory cells in liquid media (+kinetin) no contamination. (ii) chicory cells in liquid media (− kinetin) no contamination.c.) cells after 4 weeks of transferring into new cell suspension culture (liquid culture media) See ***Figures*** **20** *and* **21**.

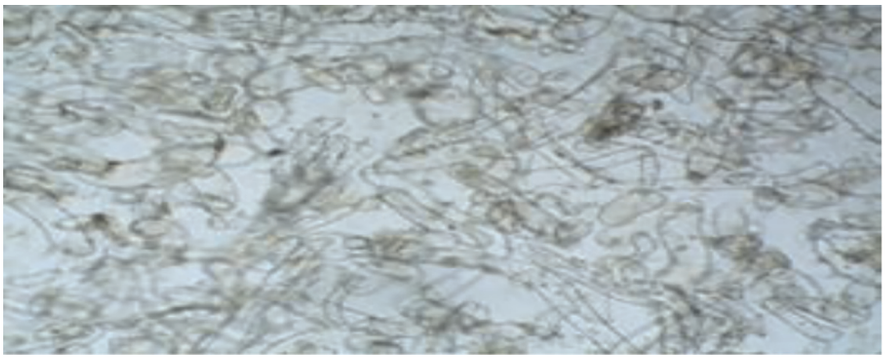


Figure 20.
(i) chicory cells in liquid media without (−) kinetin presence of contamination.

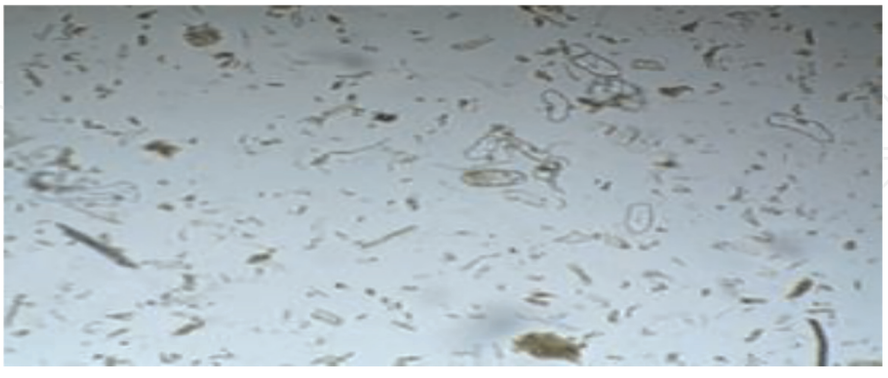


Figure 21.
(i) chicory cells in liquid media with (+) kinetin absence of contamination.

effect of plant regulators on plant cells. In addition, external factors, such as light intensity and temperature, could have affected the slow development of cells in the media with Kinetin. Since temperature and light play essential roles in plant growth, this agrees with the findings of Yildiz [57] elaborated on the influence of control equipment causing the culture room to be overheated resulting from temperature

changes, which causes stress to plants enabling very low or no success in the growth rate.

3.6 Wet and dry matter determination

Two Factors – (I) Liquid culture media with (+) Kinetin and (II) Liquid culture media without (−) Kinetin were used to determine the outcome for wet and dry weight analysis of callus of chicory culture in liquid media.

3.6.1 Liquid culture media with kinetin (+ kinetin)

The result, as shown in **Figure 22**, can be deduced that the wet weight of samples A (0.81714), B (0.98178), and C (0.92866) had a close range in the values obtained for the wet weight and that of the dry weight for all samples showed relatively very low variation during drying hence, the dry weight between the samples is approximately the same. The sample with the lowest weight after drying was sample A (0.16974), next to it was sample C (0.17406), then B (0.17978). Thus, the upsurge in dry weight was due to cell splitting 14 and new material synthesis (see **Table 4**) [104, 105].

3.6.2 Liquid media without kinetin (− kinetin)

The result in **Figure 23** shows that the wet weight of samples A (0.97604), B (0.97378), and (1.16438) varied. Thus, making the dry weight fall within the same range. Nevertheless, A (0.17284) had the most negligible value after drying; next were B (0.17808) and C (0.18568), respectively. The increase in the wet weight before drying

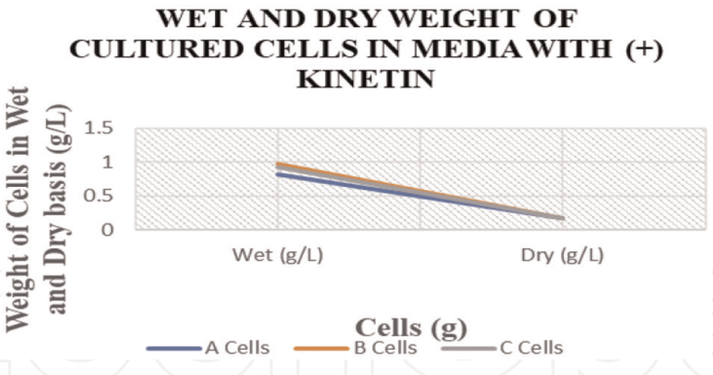


Figure 22.
Wet and dry matter determination of liquid culture media with kinetin (+ kinetin).

	Weight of Empty Filter paper	Volume of Sample	Weight of Paper + Wet Cells	Weight of Wet Cells	Weight of Dried Cells + Paper	Weight Dried Cells
	(g)	(L)	(g)	(g/L)	(g)	(g/L)
A	0.2098	0.005	0.8591	0.81714	0.2117	0.16974
B	0.2171	0.005	1.0252	0.98178	0.2232	0.17978
C	0.2137	0.005	0.9714	0.92866	0.2168	0.17406

Table 4.
Wet and dry matter determination of liquid culture media with kinetin (+ kinetin).

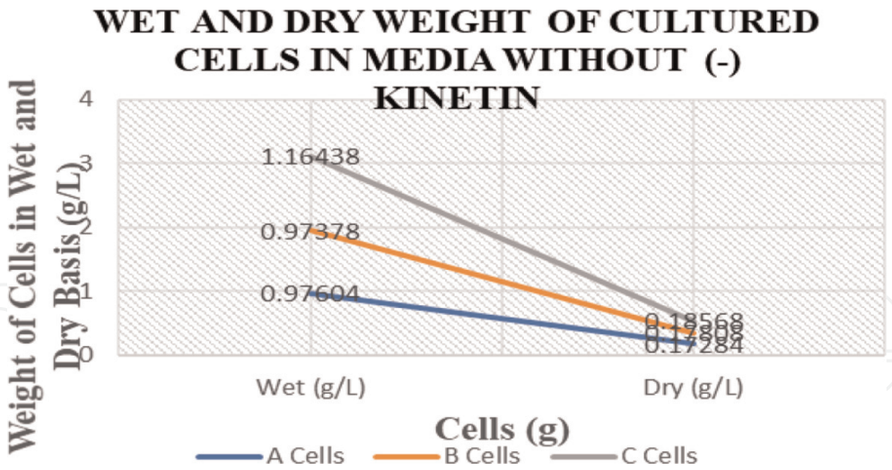


Figure 23.
Wet and dry matter determination of cells in liquid culture media without kinetin (− kinetin).

	Weight of Empty Filter paper	Volume of sample	Weight of Paper +Wet Cells	Weight of Wet Cells	Weight of Dried Cells + Paper	Weight Dried Cells
	(g)	(L)	(g)	(g/L)	(g)	(g/L)
A	0.2048	0.005	1.0170	0.9760	0.2138	0.1728
B	0.2096	0.005	1.0157	0.97378	0.2200	0.1781
C	0.2181	0.005	1.2080	1.1644	0.2293	0.1857

Table 5.
Wet and dry weight of cultured cells in media without kinetin (− kinetin).

resulted from external factors, leading to water absorption and turgor pressure of cells from the enlarged cell, which agrees with the study of Dale [106]. At the same time, the rapid decrease in moisture during drying could be as a result of the osmotic stress on the cells, which can cause moisture to escape from the cells [107] (see **Table 5**).

4. Technological part

4.1 Material balance

4.1.1 Raw materials for media preparation

Sucrose, solution of iron chelate, macronutrients, micronutrients, vitamins, Kinetin, 1-naphthalene acetic acid (NAA), dichlorophenoxyacetic acid, and distilled water.

1. **Sucrose** is a non-reducing disaccharide made of glucose and fructose linked via their anomeric carbons. It is a disaccharide formed by glucose and fructose units joined by an acetal oxygen bridge from the hemiacetal of glucose to the hemiketal of the fructose. It plays a role as an osmolyte, a sweetening agent, a human metabolite, an algal metabolite, a *Saccharomyces cerevisiae* metabolite, and an *Escherichia coli* metabolite. It is obtained commercially from sugarcane, sugar beet (*Beta vulgaris*), and other plants and is used extensively as a food and a

sweetener. The molecular formula of sucrose is **C12H22O11,** and the molecular weight is **342.3 g/mol**.

2. **Solution of Iron Chelate:** This compound helps maintain the pH in the culture media for plants and corrects an iron deficiency in plants. The molecular formula is **C20H14FeN2O2**$^{+2,}$ and the molecular weight is **370.2 g/mol**.

3. **Macronutrients** are nutrients required by plants in more significant quantities, and they include: Nitrogen (N), Potassium (K), Calcium (Ca), Magnesium (Mg), Phosphorous (P), and Sulphur (S)

4. **Micronutrients** are those nutrients required by plants in fewer quantities, such as Chloride (Cl), Iron (Fe), Boron (B), Manganese (Mn), Zinc (Zn), Copper (Cu), Molybdenum (Mo), and Nickel (Ni)

5. **Vitamins**: Plants need vitamins for their growth, and examples of such vitamins include: Vitamin B, Vitamin C, and Vitamin E. Plants may derive specific benefits from applying these vitamins.

6. **Kinetin:** This is referred to as plant hormones known as cytokinin that helps to promote cell division and plant growth. Kinetin is an adherent part of the class of 6-aminopurines that is adenine carrying a (furan-2-ylmethyl) substituent at the exocyclic amino group. It is an adherent part of furans and a member of 6-aminopurines. It has a molecular formula of **C10H9N5O** and a molecular weight of 215.21 g/mol. It was shown to naturally exist in the DNA of organisms, including humans and various plants. In contrast, Kinetin is used in tissue cultures to produce new plants.

7. **1-Naphthalene acetic acid (NAA):** A synthetic plant hormone in the auxin family and is an ingredient in many commercial plants rooting horticultural products; it is a rooting agent used for the vegetative propagation of plants from stem and leaf cuttings. It is also used for plant tissue culture. It is an organic compound with the formula **C10H7CH2CO2H**. This colourless solid is soluble in organic solvents.

8. **Dichlorophenoxyacetic acid 2, 4-D** is a member of the phenoxy family of herbicides, which include: 2, 4-D is a synthetic auxin, which is a class of plant hormones, and by itself, it is often used in laboratories for plant research and as a supplement in plant cell culture media, such as MS medium. It was a primary ingredient in Agent Orange alongside its chemically similar relative, 2, 4, 5-T (2,4,5-trichloro phenoxy acetic acid). Its molecular formula is **C8H6Cl2O3** or **Cl2C6H3OCH2COOH,** and its molecular weight is 221.03 g/mol.

9. **70%Ethanol/Alcohol** is an ideal solution that is strong enough to reduce microbial contamination to an extent. It was used to sanitise the hands and working area before the media preparation [108, 109].

4.1.2 Process description

Stage 1: Preparation of Culture media.

The nutrient medium for growing chicory callus was 1 liter, which contained 30 g sucrose, 5 ml iron chelate solution, 50 ml macronutrient, 1 ml vitamins, 1 ml kinetin, 1 ml dichlorophenoxyacetic acid and 2.5 ml 1-naphthalene acetic acid (NAA).

Stage 2: Autoclaving.

The plant culture media was autoclaved at 121^0 C.

Stage 3: Callus Cultivation.

Cells of Chicory were inoculated in the sterile liquid culture media under controlled parameters of 16 hours light intensity phase at 24°C and 8 hours dark phase at 18°C for 1 week.

Stage 4: Separation.

A total of 5 ml of starter was pipetted into six centrifuge tubes, and centrifugation was done for 10 mins at 5000 rpm at 15°C. Thus, the aliquot was separated, and 5 ml of sterile water was added to the supernatant and centrifugation was repeated twice.

Stage 5: Drying.

Six (6) filter papers were dried in the oven for 9 hours at 60 °C, and the empty weight of the filter paper was obtained, and the weight of the filter with the sample was also obtained. The samples were later placed in the oven for 24 hours at 60°C. The weight of the dried sample on the filter was weighed. The fresh and dry weights were determined.

4.1.3 Process block diagram to produce dried chicory

See **Figure 24**.

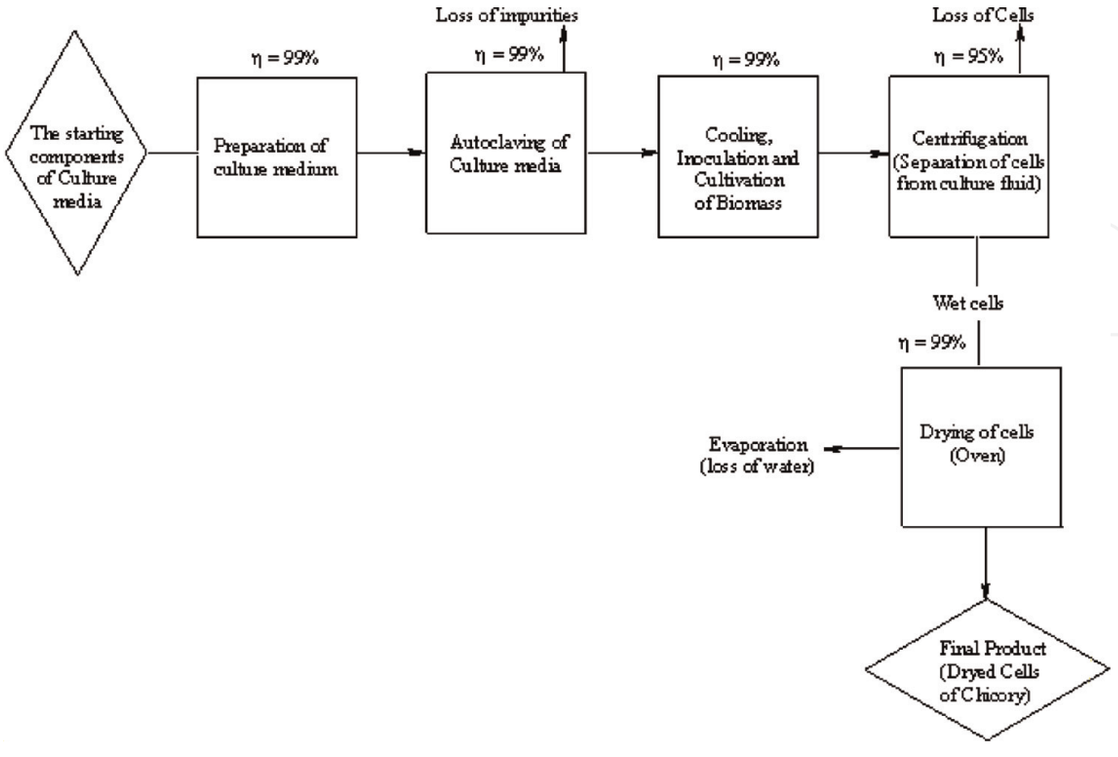


Figure 24.
Process block diagram to produce dried chicory cells.

4.1.3.1 Mass balance calculation

Characteristics of Final Product:

Form = Dried Cells Amount = 1 kg Purity = 98%.

Humidity = 1%.

Others (Impurities) = 1%.

Based on the production of 5 g of dry biomass per 1 L of culture medium (3% Sucrose).

The density of the nutrient medium - 1024 kg/m^3.

The yield of dry biomass, considering losses at the stages of cultivation, separation, and drying.

Overall yield (η) = 0.99 ∗ 0.95 ∗ 0.99 = 0.93%.

Quantity of Liquid culture media (3% Sucrose) considering losses.

Mass of culture medium = $\frac{1 * 0,001 * 1.024}{0,005 * 0.93}$ = 220.22 kg.

Considering losses (preparation and sterilisation) = 220.22/0.99/0.99 = 224.69 kg.

Mass of sugar needed = 224.69 ∗ 0.03 = 6.74 kg.

Iron chelate = 0.005 * 224.69 = 1.12 kg.

1-Napthalene acetic acid = 0.0025 * 224.69 = 5.12 kg.

Kinetin = 0.001 *224.69 = 0.56 kg.

Dichlorophenoxyacetic acid = 0.001 *204.80 = 0.22 kg.

Macronutrients = 0.05 *224.69 = 11.23 kg.

Micronutrients = 0.001*224.69 = 0.22 kg.

Vitamins = 0.001* 224.69 = 0.22 kg.

Total = 13.82 kg.

Quantity of water was calculated as:

Amount water needed = Amount of Liquid media − All solids (All media compositions) − Sugar Amount of Water needed = 224.69–13.82 − 6.74 (sugar) = 204.13 kg.

Summary of Raw Materials needed:

Sugar = 6.74 kg All solids = 13.82 Water = 204.13 kg.

Raw Materials:

4.1.3.2 First stage: Preparation of culture media

Process Yield (η) = 99%.

See **Table 6**.

Input				Output			
RAW MATERIALS	**Content (%)**	**Weight (Kg)**		**PRODUCT**	**Content (%)**	**Weight (Kg)**	
		Tech	**100%**			**Tech**	**100%**
Water	90.85		204.13	1) M&S Media		222.44	
Sugar	3		6.74	2) Losses		2.25	
Iron Chelate	0.5		1.12				
Napthalene acetic acid	0.25		0.56				
Kinetin	0.1		0.22				
Dichloro- phenoxyacetic acid	0.1		0.22				
			11.2				

Input				Output			
RAW MATERIALS	**Content (%)**	**Weight (Kg)**		**PRODUCT**	**Content (%)**	**Weight (Kg)**	
		Tech	**100%**			**Tech**	**100%**
Macronutrient	5		3				
Micronutrients	0.1		0.22				
Vitamins	0.1		0.22				
Total=		**224.69**		**Total=**		**224.69**	

Table 6.
Mass balance preparation of culture media.

4.1.3.3 Second stage: Autoclaving

Process Yield (η) = 99%.
See **Table 7**.

Input				Output			
RAW MATERIALS	**Content (%)**	**Weight (Kg)**		**PRODUCT**	**Content (%)**	**Weight (Kg)**	
		Tech	**100%**			**Tech**	**100%**
1) M&S Media		222.44		1) M&S Media		220.22	
				2) Losses		2.22	
Total=		**222.44**		**Total=**		**222.44**	
	η = 0.99						

Table 7.
Mass balance for autoclaving.

4.1.3.4 Third stage: Cultivation of cells process: Yield (η) = 99%

Cultivation time – 336 h.
Air Consumption – 0.06 m^3/min Air Density – 1.39 kg/m^3.
Mass of Air = 336*60*0.06* 1.39 = 1681.34 kg.
See **Table 8**.

Input				Output			
RAW MATERIALS	**Content (%)**	**Weight (Kg)**		**PRODUCT**	**Content (%)**	**Weight (Kg)**	
		Tech	**100%**			**Tech**	**100%**
1) M&S Media		220.22		1) Culture fluid Including Biomass Water and Soluble/ Unsoluble components	0.2 0.8	219.42	5.28 214.14
2) Inoculum Including Media Plant cells	90 10	1.42	0.67 0.75				
				2) Air with Impurities and Losses		1683.56	
3) Sterile air		1681.34					
Total=		**1902.98**		**Total=**		**1902.98**	
	η = 0.99						

Input				Output			
RAW MATERIALS	Content (%)	Weight (Kg)		PRODUCT	Content (%)	Weight (Kg)	
		Tech	100%			Tech	100%

Table 8.
Mass balance for cultivation of cells.

4.1.3.5 Fourth stage: Cell separation

Process Yield (η) = 95%.
See **Table 9**.

Input				Output			
RAW MATERIALS	Content (%)	Weight (Kg)		PRODUCT	Content (%)	Weight (Kg)	
		Tech	100%			Tech	100%
1) Culture fluid	0.2	219.42	5.28	1) Wet cells Including		5.02	1.00
Including Biomass	0.8		214.14	Dry substance Water		0.199	4.01
Water and Soluble/				Impurities		0.799	0.01
						0.002	
				2) Liquid phase		214.40	
Total=		**219.42**		**Total=**		**219.42**	
	η = 0.95						

Table 9.
Mass balance for cell separation.

4.1.3.6 Fifth stage: Drying of cells

Process: Yield (η) = 99%.
See **Table 10**.

Input				Output			
RAW MATERIALS	Content (%)	Weight (Kg)		PRODUCT	Content (%)	Weight (Kg)	
		Tech	100%			Tech	100%
1) Wet cells Including Dry	19.92	5.02	1.00	1) Dried cells Dry	98	1.00	0.99
substance Water Impurities	79.88		4.01	substance Water	1		0.01
	0.20		0.01	Impurities	1		0.01
				2) Evaporated water			4.00
				3) Losses			0.01
Total=		**5.02**		**Total=**		**5.02**	
		η = 0.99					

Table 10.
Mass balance for drying cells.

5. Summary

The suitable sterilisation method for stevia seeds is method D, which gave 14.29% per cent contamination, while that of Chicory was method C, which gave 30% per cent contamination. However, for Bee balm and *Rhodiola krylovii,* the four methods used for sterilisation and cultivation yielded very high percentage contamination of 100%, which signifies that Bee balm and *Rhodiola krylovii* need a different method of sterilisation, and this can be concluded that each plant cell requires a specific sterilisation method for increasing yield and lowering microbial contamination. The use of growth regulators for callus formation and shoot regeneration should be used when obtaining the callus of medicinal plants. Hence, this can be seen in the cultivation process of callus of Chicory, Bee balm, and *Rhodiola krylovii,* which showed an increased growth rate and percentage contamination of 62.5% for both chicory and Bee balm and 75% while the callus of the same plants grown in media without Kinetin showed no growth. They had a very high percentage contamination of 100% for the same plant species (see **Figure 25**).

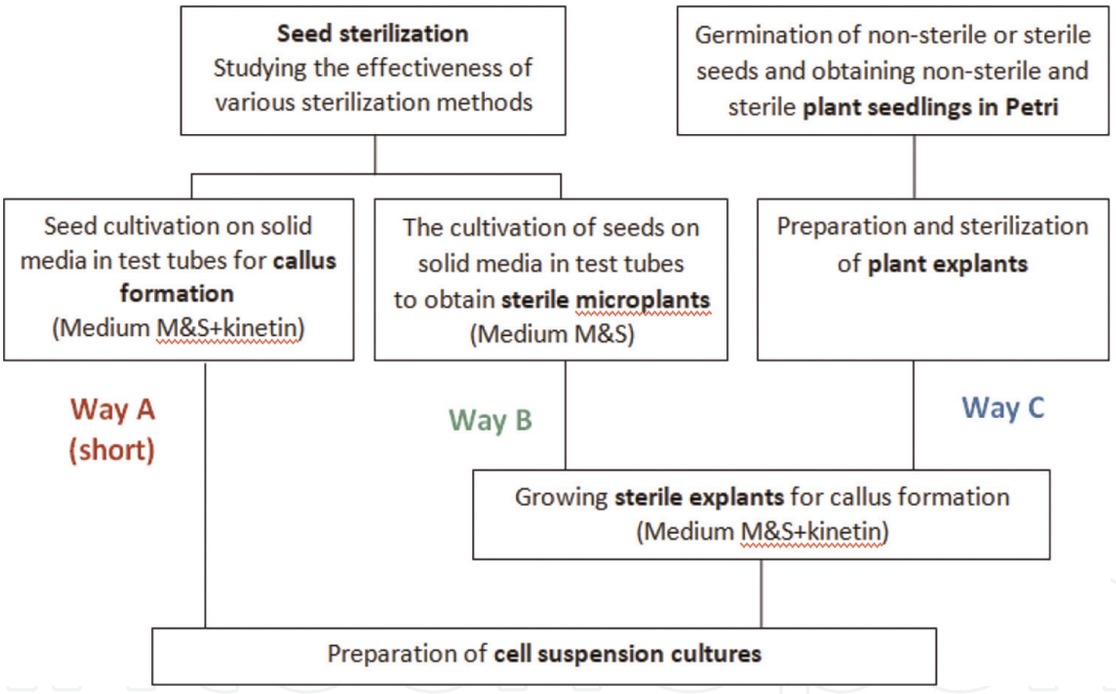


Figure 25.
Obtaining cell cultures of medicinal plants.

6. Conclusion

In a nutshell, the *in vitro* process of cultivating medicinal plants is a very tedious but delicate process that involves time, energy, proper care of the working area, and controlled environmental factors, such as temperature, humidity, moisture, and control equipment, which may alter the growing process and incur microbial contamination of plants. Thus, it was imperative to have studied the various sterilisation methods (A, B, C, and D) and the most effective method for the respective plants was used in this experiment since individual plant species have a particular method of

sterilisation that can yield good results during *in vitro* propagation of these plants. Although the issue of contamination cannot be avoided entirely during the sterilisation and cultivation process of medicinal plants, it can be significantly reduced if the parameters (pH, concentration 3% and temperature of around 10°C and 11°C) of the sterilising agent, such as NaOCl, are maintained in the required form and the pH of the media should be adjusted to around 5.8 [110]. To improve these medicinal plants' production yield and the sterilisation, cultivation process of Bee balm and Rhodiola krylovii should be studied for further research. The bioactive compounds of Chicory, Stevia, Beebalm, and *Rhodiola krylovii* produced using methods C and D, which had less percentage contamination, should be compared to the bioactive compounds of another sterilisation and cultivation method that might be used in growing the same plants elsewhere.

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Conflict of interest

The authors declare no conflict of interest.

Thanks

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