Narrative review of production of antioxidants and anticancer compounds from *Bryophyllum* spp. (*Kalanchoe*) using plant cell tissue culture

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Abstract: For centuries, plants have been widely used in traditional medicine worldwide for the treatment of many diseases. The subgenus *Bryophyllum* (genus *Kalanchoe*) have been used in ethnobotanic medicine across America, Africa and Asia. Traditionally, some formulations derived from leaves and roots of *Bryophyllum* spp. have been applied for the treatment of common illness such as coughs, fever, infections, insect bites, wounds and burns. Phenolic compounds and bufadienolides are the two major families of secondary metabolites identified in several species of the subgenus *Bryophyllum*. These compounds have gained much attention due to their associated antioxidant and cytotoxic activity, but they are synthetized by plants in fairly limited amounts. In this sense, plant tissue culture (PTC) technology provides a powerful methodology, able to overcome the limitations of low yields associated with conventional open field cultivation of medicinal plants. Several types of PTC methods are routinely employed in plant *in vitro* propagation, although micropropagation and cell culture are the most common. While micropropagation provides a reliable multiplication procedure, enabling a continuous *in vitro* production of great amounts of whole medicinal plantlets or just their organs producing the bioactive metabolites, such as their leaves and/or roots; the cell suspension culture procedure allows for the massive production of secondary metabolites using huge bioreactors. In both cases, the addition of biotic and abiotic elicitors and metabolic precursors trigger the bioaccumulation of secondary metabolites through the induction of plant defense mechanisms.

Keywords: Ethnomedicine; bioactive compounds; oxidative stress; bufadienolides; plant *in vitro* culture

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Introduction

The term "Ethnobotany" refers to the study of the practical uses of plants, which has been perpetuated over time thanks to the traditional knowledge of the local population (1). The knowledge of plants as sources of medicines, known as Ethnobotanic Medicine (2), has a long history in the treatment of many diseases worldwide (3) and it dates back about 60,000 years ago (4). Currently, the use of plants for therapeutical purposes is more extended in countries with poor economic development (5) and rural areas (6), where the modern medicine is difficult to access (7) and the indigenous knowledge about these plants is still preserved (8). According to the World Health Organization (WHO), approximately the 80% of world's population from economically developing nations depends on plants and their natural derived by-products for their primary healthcare (9). In this sense, the ethnobotanical study of medicinal plants and their uses in local regions, is not only useful for the conservation of biodiversity and cultural traditions (10), but also could serve at the starting point for the development of novel drugs (11). Among the 500,000 species of plants estimated in the world (12), only around the 5% have been studied from a pharmacological point of view (13), conferring a broad territory of unexplored plants with potential medicinal properties.

The subgenus *Bryophyllum* (genus *Kalanchoe*) comprises approximately 25 succulent species endemic to Madagascar (14) naturalized across South America, Africa and Asia (15). *Bryophyllum* spp. were widely studied in the field of Plant Science, as they are considered plant models for the Crassulacean Acid Metabolism (CAM) (16), plant cell regeneration (17) and vegetative asexual reproduction (18). However, *Bryophyllum* subgenus gained much interest in the last decades for the use of different species in traditional medicine worldwide (19-27). Concerning their properties as medicinal plants, several formulations from leaves and roots of *Bryophyllum* spp. have been used traditionally for the treatment of common illnesses such as cough, fever, infections, insect bites, wounds and burns, among others (28).

Due to therapeutic properties widely reported on *Bryophyllum* spp. (29), several phytochemical analyses were performed with the objective of determining the compounds responsible for the pharmacological potential associated to this subgenus (30). In this way, plant extracts obtained from *Bryophyllum* spp. have demonstrated to be an efficient source of analgesic (31), antidiabetic (32), anti-inflammatory (33), antimicrobial (34), antioxidant (35), antiviral (36), cardioprotective (37), cytotoxic (38), hepatoprotective (39) and sedative (40) agents. Much of the research on this subgenus has focused on a single species, *Bryophyllum pinnatum* (Lam.) Oken, due to its ubiquitous distribution and wide use in ethnomedicine worldwide (41-46). In this context, the limited knowledge about its phytoconstituents, along with its reported therapeutic effects, makes *Bryophyllum* an unexplored subgenus with a promising phytochemical potential.

On this review, we performed an overview of the peer-reviewed published literature on this topic, using the Web of Science, PubMed and Scholar Google, according to PRISMA (Preferred Reported Items for Systematic Reviews and Meta-Analyses) guidelines.

We present the following article in accordance with the Narrative Review reporting checklist (available at http://dx.doi.org/10.21037/lcm-20-46).

Bryophyllum as a source of secondary metabolites with phytochemical potential

Different authors have already identified several families of *Bryophyllum* spp. bioactive compounds, including bufadienolides, phenolic acids, flavonoids, organic salts, terpenoids and fatty acids (29,47-50). All of them are considered bioactive metabolites, biosynthesized by the induction of secondary metabolism, in response to different stimuli, collectively known as stresses (51). Therefore, these bioactive compounds are the result of the adaptative and/or defensive responses to different biotic and abiotic threats, such as infections and attacks by microorganisms, insects and herbivores (52) and drought, extreme temperatures or UV-radiation (53), respectively. Among the large number of secondary metabolites that have been reported in *Bryophyllum* subgenus, bufadienolides and phenolic compounds, are considered the two major families (28), because of two reasons: (I) they are ubiquitously distributed among all the subgenus (54); and (II) both families are responsible for the development of the bioactivities associated to these species (55). However, much attention has been paid to the bufadienolides from *Bryophyllum* sources, and several reports have focused on these compounds exclusively, as it is the case of *Bryophyllum daigremontianum* (Raym.-Hamet et Perr.) Berg. (48,56-59).

Phenolic compounds as potent antioxidant compounds

Phenolic compounds constitute the largest family of secondary metabolites with more than 8,000 compounds described, ubiquitously distributed in the Plant Kingdom (60). Due to the large number of compounds and the structural heterogeneity found on this family, a large number of subfamilies have been established (61). The great importance of phenolics as bioactive compounds, lies in their wide demonstrated antioxidant activity, developed by multiple mechanisms, acting as: (I) anti-radical agents (62), (II) modulators of antioxidant enzyme activity (63), (III) preservatives of lipid oxidation (64) and (IV) regulators of oxidative stress (65). Because of their preserving effect against oxidative stress, phenolic compounds have been reported as effective for several diseases promoted by this phenomenon, such as cancer (46), cardiovascular and neurodegenerative diseases (66) and diabetes (67). Besides antioxidant activity, phenolic compounds present additional bioactivities, acting as anti-inflammatory (68), antimicrobial (69), antiviral (45) and cytotoxic (70) agents, which may develop beneficial effects on the above-mentioned diseases.

In the case of the medicinal *Bryophyllum* spp., two major subfamilies of phenolic compounds has been described: phenolic acids and flavonoids. Caffeic acid, ferulic acid and protocatechuic acid are the major representatives of phenolic acids, as reported in *B. daigremontianum* and *B. pinnatum* (49) and flavonol glycosides, including kaempferol and quercetin glycosides, such as more abundant compounds of flavonoids (48,71).

The antioxidant activity of *Bryophyllum* extracts has been already assessed in terms of radical-scavenging activity (72,73) and the inhibition of lipid peroxidation (74). Particularly, the use of these extracts as preservatives of fish oil emulsions was recently assessed, leading to the prevention of omega-3 acids oxidation (74). Furthermore, the use of environmental-friendly procedures for the purification of phenolic compounds from *in vitro*-cultured *Bryophyllum* extracts, using activated carbon was also reported recently (75). In conclusion, *Bryophyllum* subgenus can be considered as a promising source of phenolic compounds and the PCT as new procedures which allow the large-scale production of those extracts.

Bufadienolides as potent anticancer compounds

Bufadienolides are secondary metabolites belonging to the cardiac glycosides family (76). Chemically, these secondary metabolites are polyhydroxy C-24 steroids characterized by the presence of a six-membered lactone ring at the C-17β position (77). Their synthesis is very complex, and it is known that their structures and conformation may play an important role in determining the potency of their biological activity (78). Some bufadienolides, such as bersaldegenin-3-orthoacetate and kalandaigremosides, are restricted to specific organs on discrete species, being identified in the leaves and roots of *B. daigremontianum*, respectively (48,79); meanwhile other bufadienolides, like bryophyllin derivatives, are ubiquitously distributed across *Bryophyllum* subgenus (58).

As cardiac glycosides, bufadienolides act as Na^+/K^+-ATPase inhibitors at the myocardial tissue, promoting cardiotonic effects and used in pharmacology for this purpose (80). However, several complications arise from the application of these compounds, since the overdosage may lead to a significant cardiotoxicity (81), thus causing a limitation for their therapeutic application. In fact, it has been reported that the accidental consumption of *Bryophyllum* spp. by cattle, causes severe cardiac symptoms leading to death (82,83). In order to overcome their inherent toxicity, synthetic analogues of these compounds are being developed to ensure their safety on the administration of bufadienolides-derived drugs in humans (84,85).

Nonetheless, besides their role as cardiotonic agents, bufadienolides gained much attention because of their associated cytotoxic activity, by the promotion of several mechanisms, including: (I) the induction of apoptosis and autophagy of malignant cells; (II) the arrest of cell cycle, tumor invasion and metastasis; and (III) the modulation of cancer-related intracellular signaling pathways (28,78). A wide variety of bioactivities, have been associated to bufadienolides extracted from *Bryophyllum* sources. For instance: (I) bersaldegenin-1-acetate has been proved to be cytotoxic against astrocytoma U-373, breast MCF-7, colorectal HT-29, glioma Hs683, lung A-549, melanoma SKMEL-28 and prostate PC-3 cancer cell lines and antiviral against Epstein-Barr virus (58,79,86,87); (II) bersaldegenin-1,3,5-orthoacetate was reported as effective against several cancer lines such as adenocarcinoma HeLa, ovarian SKOV-3 and melanoma A-375, and as anti-influenza, insecticidal and sedative agent (48,58,59,86,87); (III) bersaldegenin-3-orthoacetate showed a cytotoxic effect against MCF-7, NCI-H460 and SF-268 cancer lines (48,77); (IV) bryophyllins A–C were shown to be effective against several cancer lines such as gastric cancer KB and A-549, and antivirals against Epstein-Barr virus and human immunodeficiency virus (48,57,58,77,86,87); (V) daigremontianin was proved to exert a cytotoxic effect against different cancer cell lines such as MCF-7 and A-549, acting also as anti-influenza, insecticidal and sedative agent (57-59,88); and (VI) kalantubosides A and B demonstrated a cytotoxic effect against HL-60 leukemia cells (76).

The knowledge derived from the possible use of the secondary metabolites identified in *Bryophyllum* spp. as phytosanitary products has aroused great interest in their valorization by traditional medicine, but also by the biopharmaceutical industry. This carries the risk of depletion of wild plants in order to obtain sufficient quantities of plants to satisfy the demand for natural compounds. In this sense, the implementation of new and highly efficient systems of plant propagation will be required to increase the amount of plant raw material and adjust it to market demand, which would allow exploring all the phytochemical potential of the *Bryophyllum* subgenus in a sustainable way. In this regard, the use of plant tissue culture (PTC) technology could increase the biotechnological production of these compounds, without putting wild populations of *Bryophyllum* at risk. The use of this technology is reviewed below.

PTC as a biotechnological system for the production of secondary metabolites with phytochemical potential

Since the beginning of agriculture during Neolithic period, humanity has had a long history of dependence on plants: an early period, for food and nutrition purposes, and a late period in which other purposes, such as medicine, were established (89). Currently, the great global demand of medicinal plants for different commercial purposes and their derived products (90), makes of crucial importance the development and implementation of efficient approaches with the aim to obtain great amounts of plant material (91). During last decades, Plant Biotechnology has focused on the development of new strategies for the industrial production of plant-derived products (92). In this context, PTC technology provide a powerful way, able to overcome the difficulties and disadvantages associated to conventional agricultural production of secondary metabolites (93). For instance, PTC prevents from challenging environmental conditions, by conferring a reliable system with controlled growing conditions (94). Additionally, taking advantage of plant cell totipotency, PTC makes possible the establishment of different culture types, including the culture of plant organs, tissues, cell, protoplasts, but also plantlets (94). This fact, facilitates the scalability of cultures, thus promoting the large-scale production of bioactive compounds (95). In all cases, the development of axenic cultures is required in order to preserve the integrity and viability of the cultured materials, thus assuring the absence of pathogenic microorganisms (96). However, the implementation of PTC methodology requires the application of specialized knowledge and technologies, thus increasing its investment (97,98).

Thanks to the great applicability of PTC, a detailed overview of the current aspects of PTC will be addressed along this section, with particular attention to the methodology applied to *Bryophyllum* spp.: plant *in vitro* propagation (micropropagation) and cell culture.

Micropropagation

Because of its advantages as a multiplication system over conventional plant macropropagation methods, micropropagation has become an important platform for the exploitation of many plant species (99). Some of the advantages offered by micropropagation are: (I) a continuous annual production due to their independence of seasonal changes (100); (II) controlled culture conditions, making possible the adjustment of most of the factors involved in growth and multiplication, such culture media (nutrients, plant growth regulators, vitamins, sugars, etc.) and growth conditions (temperature, light intensity and photoperiod, etc.) (101); (III) the possibility to produce clones from plants difficult to propagate vegetatively and to choose desirable specific traits (102); and (IV) the less energy and space required to maintain a large-scale stock of plant material (103).

Among the widely available micropropagation methods, they can be classified into: (I) organogenesis by meristematic tissue of apical and axillary buds (104-106); (II) organogenesis by adventitious shoots and roots (107-109) including: direct adventitious organogenesis (106) and indirect adventitious shoots from callus (109); and (III) somatic embryogenesis including direct embryogenesis (110) and indirectly-initiated somatic embryos from callus (111).

Despite of the whole diversity of micropropagation methods, this technique involves some common steps along the multiplication process. An initial stage (stage 0) is essential to select healthy mother plant material, followed by a second stage (stage I) to initiate the establishment of the culture in which plant material is disinfected for the establishment of axenic cultures. After that (stage II) it takes place a stage of multiplication for obtaining new buds, propagules or embryos formation, capable of giving rise to complete plants. Sometimes, depending on the final objective, the multiplication stage can be followed by rooting (stage III) and acclimatization (stage IV), prior to the transference of micropropagated plants into natural environment (*ex vitro*) (112).

Concerning *Bryophyllum* spp. micropropagation and plant regeneration protocols, the available published research is limited. Nevertheless, some authors have achieved the multiplication of some *Bryophyllum* spp. by using Murashige and Skoog (MS) formulation (113-116). MS medium is considered the most relevant formulation designed to date, being considered as universal medium for multiple PTC applications with more than 82,000 citations on literature. Nevertheless, as individual species show specific nutritional requirements, MS may cause several physiological disorders in some cases (117,118), and MS modifications are usually applied (119). In the case of *Bryophyllum* spp., the reduction in macronutrient concentration to half-strength, improves the *in vitro* growth and multiplication in *B. daigremontianum*, *Bryophyllum* × *houghtonii* D.B.Ward and *Bryophyllum tubiflorum* Harv. species (73-75). On the other hand, the organogenetic responses in *Bryophyllum* spp. by the addition of several concentrations of plant growth regulators have been reported by several authors, by focusing on the design of indirect organogenesis protocols (115,116,120). Furthermore, it was recently reported that cytokinin 6-belzylamonipurine (BAP) drives the organogenetic process on *B. tubiflorum* showed prevalence for indirect organogenesis via callus formation, whereas *B. daigremontianum* and *B.* × *houghtonii* developed organogenetic responses from direct organogenesis (121).

Plant cell suspension cultures (PCSCs)

In recent years, the commercial importance of secondary metabolites resulted in a growing interest of the enhancement in the production of bioactive plant molecules, through different plant biotechnology methodologies (122). Plant cell culture systems have been successfully applied for the production of secondary metabolites (119). This way, PCSCs have emerged as a reliable biotechnological system, offering a robust productive system, in comparison with the production and extraction of bioactive molecules from whole plants and tissues (123). PCSCs are established from the transference of callus-derived cell aggregates to liquid medium, thus enabling their promotion to large-scale systems, such as bioreactors (124). However, PCSCs methodologies, is not exempt of their own limitations and difficulties. The fact that plant material in this type of culture is based on dedifferentiated cells, may cause genetic instability and somaclonal variation, which can result in epigenetic changes (125). Moreover, concerning productivity, PCSCs are composed by heterogenous cell populations, showing high-yielding, low-yielding and non-producing cell lines, which may negatively affect the production of secondary metabolites (95).

As a solution, different strategies can be applied to PCSCs, with the aim of improving the production of secondary metabolites, such as: (I) the selection of stable and high-yielding cell lines (126); (II) the implementation of two-phase culture systems to prevent cell auto-toxicity, by the accumulation of produced secondary metabolites (127); (III) the optimization of cell culture conditions (nutrients, plant growth regulators, temperature, light, etc.) (128); (IV) the addition of elicitors and metabolic precursors, to induce the biosynthetic pathways involved in the production of secondary metabolites (129); (V) the use of metabolic engineering to overexpress target pathways, inhibiting competing pathways and preventing the degradation of the final product (92) and; (VI) the selection of appropriate operational aspects and configuration of bioreactors, according to the nature of cultured cells (130).

Among the multiple strategies for the enhancement of the production of secondary metabolites, elicitation has been traditionally used for this purposes (131), as widely reported in countless successful cases (132). As defined by Narayani and Srivastava (2017), an elicitor is a "*physical or chemical agent capable of inducing or stimulating defense response in plant/cell tissues via production of secondary metabolites*" (133). Depending on their nature, elicitors are classified as biotic, deriving from biological sources (bacteria, fungal, algae, plant, animal-derived compounds), or abiotic, if are derived from non-biological sources (physical agents, such as UV-radiation, temperature, light, salinity, etc.; or chemical agents such as heavy metals) (134). Thus, the addition of elicitors into culture medium is a potent biotechnological strategy, which can lead to an increase in the accumulation of secondary metabolites in cell suspension cultures (135), through the induction of plant defense mechanisms leading to the biosynthesis of secondary metabolites.

Concerning PCSCs from *Bryophyllum* spp., very limited information is available. PCSCs from *B.* × *houghtonii* were subjected to elicitation with cyclodextrins (73). The results demonstrated that only 7 days were needed to achieve a stable cell growth and production of phenolic compounds. Moreover, it was shown that the elicitation by cyclodextrin addition into culture medium, improved the production of phenolic compounds and their associated radical scavenging activity (73).

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