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Original Article

The Impact of Elicitor (chemical and physical) and Explant Source on the Synthesis of Silymarin through Suspension Culture of *Silybum marianum*: A Meta-Analysis

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ABSTRACT

Silybum marianum, commonly known as milk thistle, is renowned for its bioactive compounds, particularly silymarin, which holds significant potential for its neuro-protective, antioxidant, anticancer, antidiabetic, antiviral, antihypertensive, anti-inflammatory, photoprotective, and hepatoprotective properties. The investigation of these factors and their interplay within the context of silymarin biosynthesis is anticipated to provide valuable insights into optimizing production processes for pharmaceutical and nutraceutical applications. In the present context, a compilation of data has been undertaken, followed by a meta-analysis, aimed at investigating the influence of elicitor (chemical and physical) and explant source (cotyledon, hypocotyl, and leaf) on silymarin production within the suspension cultures of *S. marianum*. Data was gathered on treatment means and sample sizes (n) concerning the production of silymarin under both control and elicited conditions. The effect size was computed using the natural logarithm of the response ratio (lnRR) as a metric to gauge the influence of elicitor treatments on silymarin production when compared to the control.

Keyword: Cell culture, Meta-analysis, Milk thistle, Secondary metabolites

INTRODUCTION

Silybum marianum, commonly known as milk thistle, is a medicinal plant that belongs to the Asteraceae family [1]. The primary bioactive constituent found in this plant is silymarin, which comprises structurally related flavonolignans, flavonoids, and various other compounds [2]. Silymarin serves as a potent antidote for counteracting hepatotoxicity caused by various toxins, including ethanol and psychotropic drugs. It effectively mitigates oxidative stress and the subsequent cytotoxic effects, thus safeguarding the integrity of liver [3- 4]. Furthermore, it offers protection against doxorubicin-induced cardiac adverse effects through its antioxidant, anti-inflammatory, and anti-apoptotic properties, among other mechanisms [5]. Additionally, silymarin finds utility as a neuro-protective, antioxidant, anticancer, antidiabetic, antiviral, antihypertensive, immunomodulatory, anti-inflammatory, photoprotective, and detoxification agent [2]. The escalating pharmaceutical demand for silymarin has underscored the need to investigate its production via biotechnological means. *In vitro* cultivation of medicinal plants presents a viable solution to challenges associated with conventional open-field production [6]. Various in vitro techniques are currently employed to enhance the content of secondary metabolites through utilization of elicitors, alterations in environmental conditions, and adjustments to the composition of the growth medium [7]. Among different techniques used in biotechnology, cell suspension cultures are prominently employed for industrial applications due to their uniformity, which leads to consistent production, rapid growth, and easy scalability for biomass generation. Elicitation, as one of the most efficacious techniques, is currently employed to enhance the biotechnological yield of metabolites [8, 9]. Generally, elicitors are classified as physical or chemical, biotic or abiotic, and complex or defined depending on their origin and molecular structure [10]. Abiotic elicitors include a range of chemical and physical stressors, including exposure to light and UV radiation, salts of heavy metals (such as Ag2S2O3, AgNO3, CdCl2, CuCl2, CuSO4, VOSO4, NiSO4, selenium), temperature fluctuations, osmotic stress induced by compounds like mannitol, sorbitol, sodium chloride, potassium chloride, cadmium chloride, PVP, and intracellular signaling molecules like jasmonic acid (JA), methyl jasmonate (MJ), salicylic acid (SA), acetylsalicylic acid (ASA) [11]. Several researchers classify intracellular signaling molecules and plant growth regulators as part of the group of biotic elicitors, while

others categorize them as abiotic elicitors [12]. Elicitor compounds exert physical or chemical stress on cell suspension cultures, provoking the production of stress-induced secondary metabolites that are typically not synthesized under normal conditions. The elicitation of cell suspension cultures encompasses both biotic and abiotic factors [7]. The type of explant is also influential in inducing callus and cell suspension culture. The different reactions of explant types to callus induction and cell suspension culture would be ascribed to the balance of endogenous hormones inside plant tissues [13]. Meta-analysis is the quantitative synthesis of the results of different studies. It is part of the larger field of research synthesis, which has had a tremendous impact on a wide range of disciplines especially in medicine and social sciences [14], and also in different fields of plant sciences like plant ecology [15], plant cell technology [16], plant breeding [17,18], plant genetic engineering [19,20], plant secondary metabolites [21,22].

In this context, we have compiled data and conducted a comprehensive meta-analysis to investigate the impact of elicitors (both chemical and physical) as well as explant types (cotyledon, hypocotyl, and leaf) on silymarin production within the suspension culture of *S. marianum*.

MATERIAL AND METHODS

Data Collection

For this meta-analysis investigation, we chose scientific databases including Scopus, Web of Science, Science Direct, ProQuest, and Google Scholar covering 292 records identified through database searching using keywords, 30 full-text articles, and 17 studies included in the meta-analysis from 2004 to 2021. The selected keywords encompassed terms such as "silymarin," "suspension culture," "callus," "elicitor," "explant," and "*Silybum marianum*."

Data Acquisition

In this study, we gathered data on treatment means and sample sizes (n) concerning the production of silymarin under both control and elicited conditions. Given that a single control treatment was contrasted with the remaining elicitor treatments, we utilized the adjusted variance to delineate the influence of the control treatment. In the Excel datasheet, standard deviation (SD) values were transformed into standard error (SE) (SE = SD/n−1/2). The inclusion of these studies contributed to enhancing the analytical power due to their influence being constrained by a moderate allocation of sample sizes [23].

Effect Sizes and Moderator Variables

The effect size was computed using the following equation, employing the natural logarithm of the response ratio (lnRR) as a metric to gauge the influence of elicitor treatments on silymarin production when compared to the control treatment without elicitors: lnRR = ln (silymarin production in elicitor treatments / silymarin production in control)

In the context of meta-analysis, response ratios were employed to assess the collective effect, namely the summarized or cumulative elicitor treatment/control effect size across various studies [23, 24]. The utilization of response ratios is common in agricultural meta-analyses due to their ability to provide a standardized, dimensionless representation of treatment-induced changes. To ensure a balanced analysis, a logarithmic transformation is applied, enabling an accurate equilibrium between positive and negative treatment effects across response ratios [23].

As discrete moderator variables, this study considered three explant types from trees: leaf, hypocotyl, and cotyledon, alongside two types of elicitors: chemical and physical. Physical elicitors encompassed factors such as photoperiod and light quality. While chemical elicitors included compounds like methyl jasmonate, salicylic acid, chitosan, melatonin, and silver.

Statistics

Meta-analyses employing random-effects models were conducted for each of the categorical independent variables through the utilization of CMA V3 software (Comprehensive Meta-Analysis Version 3; Englewood, NJ).

Heterogeneity and Publication Bias

The assessment of inconsistency was accomplished using I2 from the fixed effects model. The statistic Q, referred to as the chi-squared statistic, was employed to evaluate the statistical heterogeneity within the context of meta-analyses, with df indicating its degrees of freedom [25]. If the I2 index proved to be insignificant, the fixed effects model was adopted. Sensitivity analysis was utilized to identify any effect sizes that might not fit well within the meta-analysis. In cases where outliers and extreme effects were identified and subsequently removed, the analysis was then repeated.

Within this meta-analysis, a statistical measure known as the classic fail-safe N was employed to explore potential publication bias. If such bias was detected and the reported findings were non-significant, the results of that particular study were excluded from the meta-analysis. It's worth noting that if there is no publication bias, the graph illustrating the data tends to be symmetrical, and the amount of variation around the intervention effect size decreases as the sample size increases.

RESULTS

This meta-analysis integrates data from 10 individual studies, resulting in a z-value of 4.535 and a corresponding two-tailed p-value of 0.00001. The fail-safe N stands at 44, indicating that 44 additional 'null' studies would be required to raise the combined two-tailed p-value above 0.050. In this instance, Kendall's tau b (adjusted for potential ties) equates to -0.222, accompanied by a one-tailed p-value (recommended) of 0.185 or a two-tailed p-value of 0.371, utilizing a continuity-corrected normal approximation. Moreover, the intercept (B0) is calculated as 5.43880, accompanied by a 95% confidence interval of (-8.685, 19.563), where t equals 0.887 and df equals 8. The recommended one-tailed p-value stands at 0.200, while the two-tailed p-value is 0.400.

The effect of both elicitor and explant source factors on silymarin production

Considering the substantial heterogeneity observed in the effect sizes across research studies (Q-value = 1261.6, P-value = 0.000, I2 = 99.25), the random-effects model was chosen for analysis. This decision was motivated by the understanding that variations in independent and dependent variables' statistical relationships can result from the influence of moderating variables. After conducting sensitivity analysis within the random-effects model (Figure 1), the response ratio for the elicitor relative to the control was determined to be 35.8% (28.59-43.14) (P-value = 0.39). Consequently, the results suggest that elicitor and explant source treatments lead to a 39% increase in silymarin production within the suspension culture, as compared to the control.

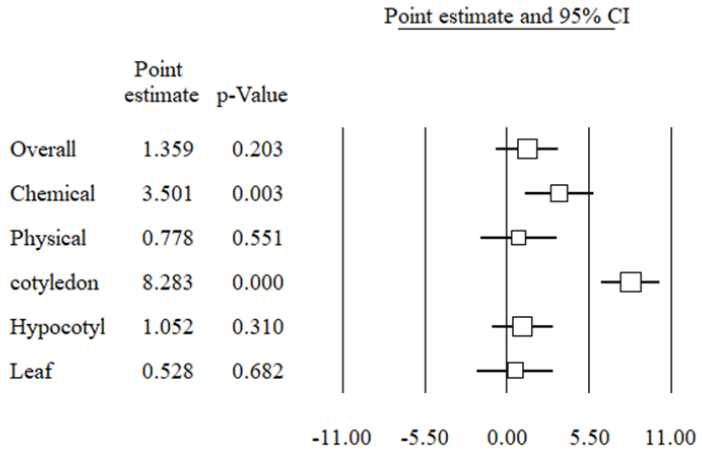

Fig. 1 Effect sizes for silymarin production are depicted, with error bars denoting the 95% confidence intervals (CI). A vertical line is included to represent an effect size of zero. The impact of elicitors on silymarin production was deemed significant when the 95% CI of the effect size for a particular variable did not intersect with zero.

The Effect of Chemical Elicitor on Silymarin Production

Owing to the considerable heterogeneity observed in the effect sizes across research studies (Q-value = 218.4, P-value = 0.000, I2 = 98.62), the random-effects model was selected for analysis. This choice is attributed to the understanding that variations in effect sizes can be influenced by moderating variables. In relation to the impact of chemical elicitors on silymarin accumulation in suspension culture, a noteworthy increase of 250% (237.39-

262.75) compared to the control was identified (Figure 1). According to the results obtained from the research, chemical elicitors such had a noticeable effect on increasing secondary metabolites.

The Effect of Physical Elicitor on Silymarin Production

Considering the significant heterogeneity observed in effect sizes across different research studies (Q-value = 547.3, P-value = 0.000, I2 = 99.63), the adoption of a random-effects model for analysis was deemed appropriate. This choice is rooted in the understanding that variations in effect sizes could arise due to the influence of moderating variables. In relation to the influence of physical elicitors on silymarin accumulation within suspension culture, a notable decrease of 22% (-38.59 to 5.78) in comparison to the control became evident (Figure 1).

The Effect of Cotyledon as an Explant on Silymarin Production

Given the limited heterogeneity found in the effect sizes across the research studies (Q-value = 0.841, P-value = 0.359, I2 = 0), the decision was made to employ the fixed-effects model for analysis. This choice is based on the understanding that effect size variations might be minimal due to the absence of moderating variables. With regard to the impact of cotyledon explants on silymarin accumulation within suspension culture, a notable and significant increase of 7 times compared to the control was evident (Figure 1).

The Effect of Hypocotyl as an Explant on Silymarin Production

Owing to the considerable heterogeneity observed in the effect sizes across the research studies (Q-value = 59.35, P-value = 0.000, I2 = 93.26), the random-effects model was selected for analysis. This choice is based on the understanding that effect size variations may be influenced by moderating variables. Regarding the impact of using hypocotyl as an explant on silymarin accumulation within suspension culture, a significant increase of 5.1% (-0.20 to 10.58) compared to the control was evident (Figure 1).

The Effect of Leaf as an Explant on Silymarin Production

Considering the notable heterogeneity in the effect sizes across the research studies (Q-value = 27.52, P-value = 0.000, I2 = 96.36), the random-effects model has been employed for analysis. This decision is informed by the understanding that variations in effect sizes may be influenced by moderating variables. In terms of the impact of using hypocotyl as an explant on silymarin accumulation within suspension culture, a substantial and significant decrease of 47.2% (-63.09 to 31.36) compared to the control was evident (Figure 1).

DISCUSSION

The biosynthetic pathways of secondary metabolites can indeed be modulated by various factors, including cultivation conditions, genetic transformation, and the use of elicitors. Moreover, alterations in membrane permeability can be achieved through the application of chemical or physical treatments [26]. The application of elicitors is widely recognized as one of the most significant approaches for enhancing the synthesis of secondary metabolites in medicinal plants [27]. Abiotic elicitors are substances that originate from non-biological sources. Abiotic stress can result from either chemical or physical factors [28]. Furthermore, elicitors are recognized for their capacity to enhance the qualitative and quantitative accumulation of secondary metabolites. This enhancement is attributed to the increased induction of enzymatic pathways responsible for the synthesis of phytochemicals [29].

In this article, in the first step, the effect of chemical and physical elicitors (together and separately) compared to the control conditions, in which no elicitors were used, in cell suspension culture on silymarin production was subjected to meta-analysis. Such improvement in silymarin accumulation in *in vitro* culture of *S. marianum* has been recorded due to different elicitors (chemical and physical) which is a confirmation of the meta-analysis done in the present study [30- 37] .The following section will provide concise summaries of scientific reports focusing on the impact of chemical and physical elicitors in augmenting secondary metabolites, with a particular emphasis on silymarin, in the suspension culture of *S. marianum*. The use of exogenous jasmonic acid (JA) and methyl jasmonate (MJ) triggers the creation of reactive oxygen species (ROS) and subsequent defense mechanisms in cultured cells and organs. This application also initiates signal transduction and the activation of multiple defense genes, resulting in the accumulation of secondary metabolites [38]. Additionally, many scientific reports have discussed the application of methyl jasmonate, either alone or combined with other stimulating agents, to enhance

silymarin production in *S. marianum* cell cultures [39- 42]. Salicylic acid is recognized as a critical factor in initiating various defense mechanisms in plants and can serve as a stimulant for the production of secondary metabolites [43]. The application of salicylic acid as a stimulant has been found to increase the production of silychristin, silydianin, and flavanolignans in cultured cells of *S. marianum* [40, 41]. Chitosan, a polycationic polymer consisting of b-1, 4 linked D-glucosamine, serves as an antifungal agent. It accomplishes this by eliciting the production of phytoalexin and pathogenesis-related proteins [44-46]. Furthermore, it has been documented that chitosan can enhance the accumulation of silymarin in milk thistle callus cultures [40, 47]. The introduction of Ag+ has been identified as an inhibitor of the ethylene signal transduction pathway [48]. Notably, the positive impact of Ag+ on production, particularly its role in enhancing silymarin accumulation in the cell suspension culture of milk thistle, has been also reported in previous studies [49, 50].

In the ongoing investigation within this research study, a meta-analysis was conducted to examine the influence of distinct explants, namely hypocotyl, cotyledon, and leaf, on the synthesis of silymarin in a cell suspension culture of *S. marianum*. The findings revealed that all three explants demonstrated suitability for generating callus tissue and facilitating the subsequent cell suspension culture for silymarin production. However, it was observed that, among these three explants, cotyledon proved to be the most effective, exhibiting a sevenfold enhancement in silymarin production in comparison to the control. Achieving high concentrations of secondary metabolites necessitates not only optimizing supportive factors such as media components and physical parameters (pH, temperature, light, etc.) and the inclusion of plant growth regulators, but also entails a critical consideration: the selection of source explants as inoculums. The choice of explants rich in secondary metabolites is paramount for achieving elevated levels of these compounds [51]. The literature review further substantiates the pivotal role of explants in the induction of callus and the synthesis of secondary metabolites within cell suspension cultures across various plant species. Notable examples include the production of rosmarinic acid, naringin, epicatechin, thymol, and carvacrol in *Dracocephalum polychaetum* Bornm and *Dracocephalum kotsc*hyi Boiss [52], as well as the generation of total polyphenols and total flavonoids in *Citrus aurantium* [53]. Phenolic and flavonoid compounds were also produced in *Lycium schweinfurthii* [54], while quercetin and kaempferol were synthesized in *Melia azedarach* [55]. Additionally, the production of silymarin in *S. marianum* has been documented by several authors [56, 57].

CONCLUSION

In a summary of the conducted meta-analysis within this research, it is paramount to underscore the significance of employing chemical and physical elicitors, with a greater emphasis on the role of chemical elicitors. Furthermore, the choice of explant, particularly the cotyledon, emerges as a crucial determinant for enhancing silymarin production within the cell suspension culture of *S. marianum.*

Conflict of Interest

Authors declare that there is no conflict of interest.

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