Conclusions
5. Conclusions

It is much obvious to say that both improper and huge harvesting of *C. borivilianum*, due to high and diversified commercial importance of its secondary metabolites, have enlisted it as “critically endangered”, as mentioned in the Red List of Threatened Plants by International Union for Conservation of Nature and Natural Resources. Therefore, in the present study, the three main approaches of plant tissue culture *viz.*; micropropagation, slow-growth storage and cryopreservation were attempted for *in vitro* conservation of *C. borivilianum*.

An effective method has been developed for the rapid *in vitro* shoot proliferation of the potential drug herb, *C. borivilianum*, which facilitated shoot production at each of up to seven subculture cycles. Moreover, seven numbers of subculture cycles, *in vitro* rooting and acclimatization were completed within the duration of one year only, which is an essential factor for commercial production of an *in vitro* propagated species. Thus, *in vitro* clonal propagation has been proved as an efficient approach for disease-free plantlet production, and also to cater the day-to-day growing demand of *C. borivilianum*. Additionally, above techniques would also be helpful in replenishing the loss of natural population of aforesaid species.

Further, the *in vitro* cultures of *C. borivilianum* were successfully stored for 4 months time without any successive transfer in other medium or addition of an extra medium in the culture vessel. This slow-growth storage was accomplished under normal culture conditions, and was regenerated healthy shoots afterwards. This strategy will be helpful in preventing the loss of genetic stability of *in vitro* cultures of *C. borivilianum* by extending the time duration between subculture cycles.

In this study, a protocol for vitrification based cryopreservation of meristematic-tips of *C. borivilianum* was also worked out. Incubation of meristematic-tips on the medium comprising sucrose (120 g L\(^{-1}\)) and ABA (50 mg L\(^{-1}\)), for 2 days, unveiled better regeneration of cryopreserved samples of *C. borivilianum*, while addition of sucrose alone in the medium did not revealed any significant impact. Moreover, a little improvement in the regeneration rate of cryopreserved meristematic-tips was obtained by extending the duration of their exposure to loading solution. However, even a single regenerated meristematic-tip could produce ample number of plantlets through
Conclusions

The micropropagation protocol developed in this study. To the best of our knowledge, this is the first report pertaining to slow-growth storage and vitrification based cryopreservation of *C. borivilianum*.

*In vitro* tuberization in the micro-shoots of *C. borivilianum* was achieved following the solid and stationary liquid medium, supplemented with moderately-higher level of sucrose. However, the stationary liquid medium was found to be more efficient for *in vitro* tuberization, as it had significantly improved the number of tuber formation. These micro-tubers will later be used either for extraction of bioactive molecules or clonal propagation of *C. borivilianum*.

Diosgenin production in micro-tubers of *C. borivilianum* was influenced by both the elicitors (SA and JA), separately. Out of these two signal compounds, the lower dose of JA resulted in increased concentration of diosgenin, while, relatively higher dose of JA showed inhibitory effect on diosgenin accumulation in micro-tubers of *C. borivilianum*. Salicylic acid elicitation to micro-tubers of *C. borivilianum* also showed similar response as JA for diosgenin accumulation but was comparatively lower than estimated in the JA elicited micro-tubers. Further, the outcome of the current study may be exploited for the enhancement of diosgenin content in *in vitro* raised cultures of *C. borivilianum* which is an important bioactive molecule for pharmaceutical industries. To the best of our knowledge, this is the first report of *in vitro* diosgenin production using signal compounds in micro-tubers of *C. borivilianum*.

Conducted study has also succeeded in replacing ammonium nitrate, one of the essential components of the MS medium, by calcium nitrate, as a substitute of nitrogen source, for micropropagation of *C. borivilianum*, particularly. Thus, the scarcity of ammonium nitrate could be overcome up to an extent, which is now-a-days prohibited in most of the countries for marketing as it is popularly used in the manufacturing of explosives.