NEW METHOD FOR EXTRACTING PHYTOCONSTITUENTS FROM PLANTS

Renu Solanki* and Badri Prakash Nagori

*Lachoo Memorial College of Science and Technology (Autonomous), Pharmacy Wing, Jodhpur, Rajasthan, India

E-mail of Corresponding Author: solankirenu@yahoo.com

Abstract
The present work is in the technical field of plant extraction. More particularly, the work relates to the extraction of medicinal plants using liquid nitrogen in a closed environment. The principal objective of this work is to provide a new method of plant extraction using liquid nitrogen which is simple, safe and less time consuming as compared to the existing open method of plant extraction using liquid nitrogen. The plant used for the study was *Cynodon dactylon* (Linn.) Pers. The new method includes the steps of size reduction of the plant material, taking the plant material and solvent in culture tubes, closing the tubes properly and dipping these tubes into liquid nitrogen at below -196 °C, melting the frozen material inside the tubes and collecting the liquid extract. The new method of plant extraction avoids exposing liquid nitrogen in open environment and thus avoids the drawbacks of the available methods of plant extraction using liquid nitrogen in open environment.
Keywords: *Cynodon dactylon*, phytoconstituents, liquid nitrogen, plant extraction, close method, soxhelation, maceration, microwave assisted extraction.

1. Introduction

Plants have been used for medicinal purposes for as long as history has been recorded. Medicinal plants are plants which contain substances that could be used for therapeutic purposes or which are precursors for the synthesis of useful drugs[1]. There are between 35,000 and 70,000 plant species that have been used for medicinal purposes in the world[2]. Either the whole plant or a specific part of it (root, stem, leaf, flower, fruit, seed etc.) is extracted with suitable solvent. The extract thus obtained is formulated into preparations like ointment, cream, gel, lotion, pills etc. To carry out the extraction of plant material various methods like infusion, decoction, digestion, maceration, percolation, reserved percolation, sonication, continuous hot percolation (soxhelation) and microwave-assisted extraction are used[3].

Liquid nitrogen is nitrogen in a liquid state at a very low temperature. It is a compact and readily transported source of nitrogen gas without pressurization. Further, its ability to maintain temperatures far below the freezing point of water makes it extremely useful in a wide range of applications. Liquid nitrogen should only be stored in containers specifically designed to contain cryogenic fluids[4].

Liquid nitrogen presents an efficient way of extracting plant material without the loss of phytoconstituents due to low temperature during the extraction. In the prior art, various methods are reported in which liquid nitrogen is used in an open environment (open method) to carry out extraction of plant materials such as DNA/ RNA, cell wall, alkaloids etc. from the plant tissues[5,6]. When the plant tissue is exposed to liquid nitrogen it causes cell lysis and penetration of the solvent in the tissue becomes easy and fast.

Liquid nitrogen is reported to be quite useful in the extraction of the genetic material (DNA and RNA) from the plant tissues. To carry out DNA/ RNA extraction from plant tissues, a mechanical means of breaking down the cell wall and membrane that allows access of the solvent to the nuclear material is used. DNA/ RNA must be purified from cellular material in a manner that prevents its degradation. For this, plant tissues are ground with liquid nitrogen along with suitable solvent in open environment like in a pestle mortar (open method). It causes cell lysis and allows access of the solvent to DNA/ RNA while cellular enzymes and chemicals that may cause degradation of DNA/ RNA remain inactivated. The extracted material is then transferred to an appropriately sized tube and the liquid nitrogen is allowed to evaporate. The extract thus obtained is further processed to isolate and purify the genetic material.

Laurence D. Melton and Bronwen G. Smith described a method of isolating plant cell walls and fractionating cell wall polysaccharides[7]. The purpose of the cell wall isolation was to obtain a preparation of cell wall that was virtually free

from contamination by cytoplasmic components of the cells (e.g., membranes, nuclear material, enzymes, and starch). This was achieved through a series of steps that include selecting the plant material based on microscopic examination; cutting the tissue into small pieces and placing each piece immediately into liquid nitrogen; placing frozen pieces of tissue into a mortar cooled with liquid nitrogen; grinding tissues along with suitable solvent with a pestle mortar to form a fine powder; keep adding liquid nitrogen to the tissue to prevent thawing; grinding small amounts of tissue at a time, placing the ground material in a beaker and keeping it frozen by continuing adding liquid nitrogen. This ground material was further processed to isolate plant cell walls and to fractionate cell wall polysaccharides.

US patent 4831133 describes a method of extracting alkaloids of *Catharanthus roseus* tissue that involve the steps of grinding fresh *C. roseus* tissue in liquid nitrogen and then extracting the ground material with suitable solvents to obtain alkaloids[8].

In all of these methods liquid nitrogen is used by pouring it on an open grinding surface such as a mortar and pestle. Liquid nitrogen is a hazardous substance and exposing liquid nitrogen in an open laboratory environment is very harmful.

2. Drawbacks of available methods of plant extraction using liquid nitrogen in an open environment[9,10]:

Liquid nitrogen is a cryogenic fluid and at atmospheric pressure it boils at −196°C (−321°F). It can cause rapid freezing on contact with living tissue, which may lead to cold burns. Liquid nitrogen can spatter (possibly in eyes) while being poured. Eye or skin contact with the liquid nitrogen can cause cold contact burns, potentially resulting in loss of sight or severe skin damage. It boils immediately on contact with a warmer object or when exposed at high temperatures, thus using liquid nitrogen in warmer countries like India by the available methods is very risky. As liquid nitrogen evaporates, it will reduce the oxygen concentration in the air and might act as an asphyxiant (reduced oxygen concentration). Nitrogen is odorless, colorless, tasteless and may produce asphyxia without any sensation or prior warning. A laboratory assistant was died in Scotland in 1999, apparently from asphyxiation, after liquid nitrogen spilled in a basement storage room. Liquid nitrogen when used in open environment can not be recovered back and thus lead to substantial losses due to its excessive use. This increases cost of the experiment. Further, grinding of the plant material with liquid nitrogen may lead to the loss of many useful phyto constituents.

Thus it shows that available methods of plant extraction using liquid nitrogen are risky, cumbersome, time consuming and requires special safety precautions. Therefore, a new method of plant extraction using liquid nitrogen is required which is simple, safe and less time consuming as compared to the existing method.

3. Materials and Methods

A new method for extracting phytoconstituents from plant using liquid nitrogen was developed which avoids the drawbacks of the available methods of plant extraction using liquid nitrogen in an open environment. The new method is termed as "Phytoconstituent extraction using liquid nitrogen in the closed environment".

3.1 Plant material: The new method for extracting phytoconstituents was performed by using whole plant material of the *Cynodon dactylon* (Linn.) Pers[11-12].

3.2 Apparatus used: A stainless steel closed jar filled with liquid nitrogen called as Lindane jar was used for extracting out phytoconstituents from plant material. Nitrogen in liquid state at a very low temperature is defined as liquid nitrogen.

3.3 Defattation of size reduced fresh plant of *Cynodon dactylon:* 20 gm fresh plant of *Cynodon dactylon* was taken and cut into small pieces. Four culture tubes were taken and each tube was filled with 20 ml petroleum ether. 5 gm size reduced plant material was added in each culture tube. These tubes were closed and shaken properly. Each tube was put inside a plastic container. The container was then placed in a jar filled with liquid nitrogen called as Lindane jar for 5-10 minutes with the help of tong. Creamy fatty substance leached out of the plant material and was frozen inside the tubes. The tubes were taken out of the jar and were dipped in the warm water for 5 -10 minutes till the frozen substance inside the tubes was melted. The tubes were then rotated between hand palms to melt the frozen fatty substance completely. This process was repeated 8-10 times, till the leaching of creamy fatty substance was stopped and defattation was completed. After defattation, the marc was taken out from tubes, spreaded as a bed on a clean paper and dried for 15-30 minutes so as to completely evaporate petroleum ether from plant

surface. 19.76 gm of defatted marc so obtained was used for hydroalcoholic extraction purpose.

3.4 Hydroalcoholic extraction of defatted fresh plant of *Cynodon dactylon:* Four culture tubes were taken and each tube was filled with 20 ml of hydroalcoholic solvent (water and alcohol in the ratio of 60:40). 19.76 gm of defatted marc obtained after defattation was added into each culture tube. Similar steps were performed for hydroalcoholic extraction as were performed for defattation of plant material. At the end of the process, filtration was done to remove plant material from melted liquid extract. The filtrate was then centrifuged at 14000 rpm for 1 hour. Supernatant liquid was removed and was concentrated on a water bath.

3.5 Comparison of the yield of extraction: The yield of extraction obtained in the new method was compared with the yields of soxhelation, maceration and microwave assisted extraction methods.

3.5.1 Soxhelation method: Soxhelation is also called as continuous hot extraction[13]. 250 gm of dried powdered whole plant of *Cynodon dactylon* was defatted with 1000 ml of petroleum ether (60-80°C) by using Soxhlet apparatus. Extraction was carried out until a drop of solvent from the siphon tube when evaporated did not leave a greasy spot. It took approx. 15-16 cycles. After defattation, the marc was taken out from extractor and spreaded as a bed on a clean paper and dried till evaporation of petroleum ether. 246.95 gm of dried defatted marc obtained was used for further hydroalcoholic extraction.

246.95 gm of dried marc obtained after defattation was packed in soxhlet apparatus and was extracted with 1000 ml of hydroalcoholic solvent system (water and ethanol in a ratio of 60:40). The batch was extracted until a drop of solvent from the siphon tube when evaporated did not leave a residue or the solution in the siphon tube becomes completely colorless. Filtration was performed and the filtrate was concentrated on water bath to get the extract.

3.5.2 Maceration method: 20 gm of defatted powdered drug material was macerated with 100 ml hydroalcoholic solution in an iodine flask, shaking occasionally during the course of maceration. After 48 hours, content was filtered and filtrate was concentrated on a water bath.

3.5.3 Microwave assisted extraction method: Heating by microwaves is caused due to interaction of the radiation with the dielectric field associated with polar molecules and ions[14]. Scientific microwave oven was used to carry out extraction of *Cynodon dactylon.*

20 gm of defatted plant material was taken in a 250 ml beaker and 200 ml of hydroalcoholic solution was added to it. It was kept as such for maceration for about half an hour. The beaker was covered with a watch glass and then placed in microwave oven and it was started at 140 watts power for 5 minutes. After 5 minutes, the beaker was taken out and the contents were filtered using Whatman filter paper no. 41. The solvent was evaporated and the residue obtained was weighed. The same procedure was repeated at different watt powers (210, 280, 350, 420, 490, 560, 630 and 700) for different time durations (5, 10 and 15 minutes). The % yield in Microwave assisted extraction method at different time duration and at different microwave power obtained is shown in table 1.

Table 1: % w/w yield of extract from Microwave assisted extraction method

Power (Watt)	% w/w yield of extract at different time durations					
	5 min		10 min		15 min	
	gm	%	gm	%	Gm	%
140 (20%)	0.66	3.30	1.10	5.50	0.47	2.35
210 (30%)	0.74	3.70	1.12	5.60	0.67	3.35
280 (40%)	0.83	4.15	1.14	5.70	0.84	4.20
350 (50%)	0.90	4.50	1.15	5.75	0.90	4.50
420 (60%)	0.94	4.70	1.11	5.55	1.10	5.50
490 (70%)	1.10	5.50	1.06	5.30	0.91	4.55
560 (80%)	1.12	5.60	0.91	4.55	0.83	4.15
630 (90%)	1.14	5.70	0.82	4.10	0.66	3.30
700 (100%)	1.15	5.75	0.68	3.40	0.60	3.00

Extract obtained from each extraction method were stored in airtight container in refrigerator below 10°C.

4. Result

The overall comparative study of % yield (w/w) of extract obtained from different extraction methods is shown in table 2.

Table 2: % yields of extract obtained by different extraction methods

Parameters	Soxhelation method	Microwave-assisted method	Maceration method	Nitrogen extraction method (Closed method)
% Yield	5.72	5.75	11.85	12.65
Colour	Brown			
Consistency	Sticky semi-solid			

5. Discussion

From the results of table 2, it was found that % yield of extract was in the order as nitrogen method > maceration method > microwave-assisted method > soxhelation method. Though the yield of extract obtained by maceration method was closer to the yield achieved by nitrogen method but the maceration method is much slower process as compared to the new nitrogen method because it takes almost 7 days for extracting out completely the phytoconstituents when judged with time duration of nitrogen method which takes only 15 minutes to complete the procedure.

The new method has the following advantages over the available methods of plant extraction using liquid nitrogen in an open environment

- Simple, less time consuming and easy to perform method.
- Safe method: As liquid nitrogen is not exposed in an open environment.
- Economic method: Since liquid nitrogen is not lost during the process and can be reused again.
- No loss of phytoconstituents during the extraction process as no grinding is required.
- Loss of thermolabile phytoconstituents is avoided as it is a cold method of extraction and no heating of the plant material is required.
- Less chances of contamination of the extract with foreign matter due to close environment in the experiment.
- No chances of contamination of the extract with liquid nitrogen as there is no direct contact of the extract with liquid nitrogen.
- Suitable for warmer countries like India where exposing liquid nitrogen in an open environment may cause accidental damages due to high atmospheric temperature.
- No excessive use of liquid nitrogen.

6. Conclusion

It was concluded that the new nitrogen method for extraction would be the best suited method. Among all the methods used, the method developed as per the present work called as nitrogen extraction method in close environment is the most efficient method as it gave maximum yield at lesser time duration.

Acknowledgment

Authors are thankful to Government Veterinary Hospital, Ratanada, Jodhpur, Rajasthan for providing the necessary facilities to carry out this work.

References:

1. Fagbohun ED, Asare RR and Egbebi AO. Chemical composition and antimicrobial activities of Urena lobata L. (Malvaceae). *J Med Plant Res*. 2012; 6(12): 2256-2260.
2. Farnsworth NR, Soejarto DD: Global Importance of Medicinal Plants. The Conservation of Medicinal Plants Cambridge University Press, Cambridge, United KingdomAkerele O, Heywood V, Synge H 1991, 25-51.
3. Troy D.B. Remington The Science and Practice of Pharmacy, Edn. 21st. Lippincott Williams & Wilkins, USA; 2006. p. 773.
4. Liquid Nitrogen: Guidance for Safe Storage and Handling. The University of Queensland, Australia. http://www.uq.edu.au/ohs/pdfs/Liquidnitrogenguidance.pdf. (accessed 30 August 2012)
5. Behr S. *et al,* inventors. Plant extracts and dermatological uses thereof, US patent 20110311661, 2011 Dec.
6. Yang Z. and Karr S. inventors. The Regents of The University of California, assignee. Compositions and methods for increasing cellulose production, US patent 20100146672, 2010 June.
7. Laurence D.M. and Bronwen G.M. Isolation of plant cell walls and fractionation of cell

wall polysaccharides. In: Current Protocols in Food Analytical Chemistry, Wiley Online Library, Malden MA; 2001. pE3.1.1-3.1.23.
8. Anne E.G., Colin D.W. and Masanaru M. inventors. Allelix, Inc., assignee. Extraction of alkaloids of *Catharanthus roseus* tissue. US patent 4831133, 1989 May.
9. Student Activity: Liquid nitrogen demonstrations. Wichita State University, Kansas, USA. http://webs.wichita.edu/facsme/nitro/safe.htm . (accessed 05 July 2012)
10. Convery P. and Benigna J. Liquid Nitrogen Demonstrations. Proceedings of the 52nd Annual MSTA Conference; 2005 March 4; Detroit, MI. http://www.semcto.com/docs/Liquid_Nitrogen.pdf. (accessed 20 July 2012)
11. Nagori BP, Solanki R. Cynodon dactylon (L.) Pers.: A valuable medicinal plant. *Res. J. Med. Plant.* 2011; 5(5): 508-514.
12. Kirtikar KR, Basu BD. Indian medicinal plants. 3rd ed. New Delhi: Sri Satguru Publications; 2001.
13. Palanisamy P. *et al.* Preliminary Phytochemical Evaluations of Whole Plant Extract of D*ipteracanthus prostratus* nees, *Int. Res. J. Pharm*., 2012, 3(1): 150-153.
14. Raman G. and Gaikar V.G. Microwave-Assisted Extraction of Piperine from *Piper nigrum*. *Ind. Eng. Chem. Res.*, 2002, 41(10): 2521-2528.