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Comparison of Extraction Efficiency of Aqueous and Methanolic Extracts from Plant Tissues and Callus Cultures of *Calotropis procera* and *Calotropis gigantea*

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Abstract

Extracts of plants may contain bioactive substances which could be used as natural antimicrobial agents against many microbes. Callus from the leaf material of *Calotropis procera* and *C. gigantea* was raised on different hormonal combinations and different extracts i.e. 50% methanolic, 90% methanolic and aqueous extracts of leaf and callus were prepared. The extraction efficiency of different extracts of plant tissue and callus was calculated and compared. Callus tissue is advantageous over plant tissues for extraction of phyto-constituents for the basic reason that purification and isolation of active constituents are easier and also scale up strategies can be applied *in vitro*. Moisture content of tissue and the callus were analyzed and improved extraction efficiency was calculated after subtracting the moisture content.

Introduction

India is a biodiversity rich country. Since ancient times, plants are being used to prepare herbal remedies. *Calotropis* is a common medicinal plant with great medicinal potential which belongs to the family Asclepiadaceae. *Calotropis procera* and *C. gigantea* are two most common species of this genus. The therapeutic potential of *Calotropis procera* (Watt and Breyer-Brandwisk 1962, Kartikar and Basu 1994, Arya and Kumar 2005, Sehgal et al. 2006, Choedon et al. 2006) and *Calotropis gigantea* (Chitime et al. 2005, Wang et al. 2008, Saratha et al. 2009, Kumar et al. 2010) is very well documented. The two species are reported to contain various phyto-constituents (Akindele et al. 2017, Chandrawat and

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Sharma 2015, Shetty et al. 2015, Verma 2014, Meena et al. 2012, Agarwal et al. 2011) in all plant parts but little work has been done on phytochemical screening of the calli of *Calotropis procera* and *C. gigantea*. Callus is a potent source of phyto-constituents because laticiferous cell differentiation is minimal at this stage which is an interfering factor in plant based extractions. Callus culture can be advantageous over various plant tissues for phyto-constituent extractions. Callus cells have the advantage that cells with or without laticiferous tissue can be differentiated *in vitro*. The presence of latex causes contamination of certain unwanted compounds into pure phyto-constituents. This further leads to use of stringent purification techniques. Moreover, the differentiation of laticiferous cells can be regulated at will *in vitro* (Datta and De 1985). Additionally, literature is silent on the extraction efficiency of the plants which is a crucial step for plant based extractions. The moisture analysis is another important factor which is generally ignored. The present study deals with the comparison of extraction efficiency of tissue and callus extracts and standardization of most efficacious combination for callus culture of *Calotropis gigantea* (KUK/BOT/IPS-20) and *C. procera* (KUK/BOT/IPS-21). Aqueous and methanolic extractions were done for both the species and their moisture content was analyzed. Extraction efficiency was quantified and compared in both the species.

Materials and Methods

Leaves of *Calotropis procera* and *C. gigantea* were collected and washed thoroughly with running tap water and then with double distilled water. Ramenta from leaves were removed with cotton swab to avoid any contamination. Leaves were first air dried and then oven dried at 60ºC for 12 hrs. Dried tissues were powdered in a grinder and kept in air tight polythene bags.

To minimize the error and to calculate the extraction efficiency of the plant material, moisture content was determined using moisture balance (AND, MX-50). Observed moisture content has been shown in Table 2.

For Soxhlet extraction, tissue was wrapped in filter paper of high porosity.

To make the aqueous extract of the plant, 5 g of powdered material was mixed in 100 ml of distilled water and was stirred on a magnetic stirrer for 3 hrs. The extract was filtered and the residue was again mixed in 100 ml. of distilled water. The extraction process was performed repeating 3 cycles and about 300 ml of extract was formed.

To make 50% methanolic extract, 2 g of powdered sample were mixed with 150 ml of 50% methanol and extracted using Soxhlet apparatus at 70ºC for 50 hrs.

To make 90% methanolic extract, 2 g of powdered sample were mixed with 150 ml of 90% methanol and extracted using Soxhlet extractor at 70ºC for 50 hrs.

The two species of *Calotropis* i.e. *Calotropis procera* and *C. gigantea* were obtained from the Horticultural Training Institute, Uchani, Karnal, Haryana. The *in vitro* cultures of these species were established using only the young leaves of the plants.

Fully expanded leaves (3rd leaf from top) and juvenile unexpanded leaves were collected from healthy and disease-free plants. The explants were washed thoroughly in tap water and ramenta (hairy growth) was removed with the help of fine brush without damaging the tissue. Sterilization was done in two steps: pre- and post sterilization. Pre sterilization was done outside the laminar air flow chamber and post sterilization was done inside the chamber. Explants were washed with a liquid detergent (Teepol) (1%) for 10 min followed by thorough washing under running tap water for 5 - 10 min to remove any residue of the Teepol. These explants were then given different treatments before inoculation to minimize contamination in the culture. Sizing of explants (1 - 2 cm) was done under a laminar air flow chamber and those were finally inoculated to indifferent media. Juvenile leaves were inoculated without sizing.

The tissue culture experiment was performed using MS basal medium. The MS was supplemented with different concentrations of growth regulators, namely NAA, BAP, Kn, 2,4-D, IAA and their combinations as specified in Table 1.

Out of all these 13 combinations (MS_0 - MS_{12}), MS_8 i.e. MS_0 + NAA (2.5 mg/l) + Kn (2.5 mg/l) [coded as conc. A] and MS_{11} i.e. MS_0 + NAA (5 mg/l) + Kn (2.5 mg/l) [coded as conc. B] were found to be the best combinations for callus induction for both *Calotropis procera* and *C. gigantea.* Calli were raised in bulk using these combinations and extraction was done through Soxhlation.

Cultures were maintained at 25 ± 2ºC and provided with 16 hrs of photoperiod (3000 lux intensity).

To prepare the 50 and 90% methanolic extracts of callus, the leaf callus was picked from the medium and washed with double distilled water to remove the traces of the media. The fresh weight of the callus was taken and then it was oven dried overnight at 50ºC. After taking the dry weight, the moisture content was determined using moisture balance shown in Table 3. The dried callus was wrapped in filter paper of high porosity and extracted with 150 ml of 50 and 90% methanol, respectively using Soxhlet apparatus at 70ºC for 50 hrs.

To prepare the aqueous extracts of callus, the leaf callus was picked from the medium and washed with double distilled water. The fresh weight of the callus was determined and it was crushed in double distilled water using pestle and

mortar and raised to 100 ml. The weight and moisture content of the callus have been shown in Table 3.

Results of the experiments were analyzed using one way ANOVA (Tables 2, 3). It was found useful for determining whether there is a significant difference between the type of extraction solvent used for extraction of secondary metabolites and the value of extraction efficiency based on the type of species. SPSS (ver. 24, Chicago (IL) USA) was used for statistical analysis.

Results and Discussion

The extraction efficiency of leaf and callus of both the species i.e. *Calotropis procera* and *C. gigantea* was calculated (Tables 2 and 3). The comparison of the extraction efficiency of leaf and callus extract has been represented by the graphs (Figs 7, 8).

The extraction efficiency of the aqueous extracts of leaf of *Calotropis gigantea* was found to be maximum (6.65 ± 0.034%), followed by 90% methanolic extracts of leaf of *Calotropis procera* (4.85 ± 0.033%). The extraction efficiency was observed to be the minimum in aqueous extracts of *Calotropis procera.* The initial weight of dried sample, moisture content, weight of extract, initial extraction efficiency and final extraction efficiency of all the aqueous, 50 and 90% methanolic extracts of *C. procera* and *C. gigantea* have been compiled.

The extraction efficiency of 90% methanolic extracts of callus of *Calotropis gigantea* (on conc. B) was found to be maximum (43.86 ± 0.03%) followed by 90% methanolic extracts of *Calotropis procera* (23.86 ± 0.03%) at concentration B. It was observed to be the minimum in aqueous extracts of *Calotropis procera* and *C. gigantea* on both A and B concentrations (less than 1%). The initial weight of dried sample, moisture content, weight of extract, initial extraction efficiency and final extraction efficiency of all the aqueous, 50% methanolic extracts and 90% methanolic extracts of *Calotropis procera* and *C. gigantea* on both A and B concentrations have been compiled in Table 3.

Extraction efficiency of methanolic extracts was found to be more than aqueous extracts. In both *Calotropis procera* and *C. gigantea,* concentration A i.e. MS_0 + NAA (2.5 mg/l) + Kn (2.5 mg/l) showed higher extraction efficiency in 50% methanolic extracts but it was the highest in 90% methanolic extracts in case of B concentration i.e., MS_0 + NAA(5 mg/l) + Kn (2.5 mg/l) for both the species.

Present results have also revealed that analyzing moisture content before the tissue/callus extraction is an important and significant step in plant based extractions. Tables 2 and 3 clearly indicate that 2 - 10% moisture content still remained in the tissue and callus even after drying it.

Table 1. Showing different hormonal combinations in which callus was raised, intensity of callus and the type of callus.

Sr. No.	Type of medium	Hormonal combination	Intensity of callus	Type of callus
1	MS_0	MS basal	-	Explant discoloration
2	MS_1	MS_0 + IAA (2 mg/l)	-	Explant discoloration
3	MS_2	MS_0 + 2,4-D (2 mg/l)	+	Creamy, slow growing callus
4	MS_3	MS_0 + 2,4 -D (6 mg/l)	++	Creamy, slow growing callus
5	MS_4	MS_0 + IAA (2 mg/l) + 2, 4-D (2 mg/l)	-	Explant discoloration
6	MS_5	MS_0+ 2,4-D (5 mg/l) + Kn (2 mg/l)	+	Creamy, slow growing callus
7	MS_6	MS_0 + NAA (2.5 mg/l)	+	Creamy, green, profuse, friable callus
8	MS_7	MS_0 + NAA (5 mg/l)	++	Creamy, green, profuse, friable callus
9	MS_8	MS_0 + NAA (2.5 mg/l) + Kn (2.5 mg/l)	+++	Creamy, green, profuse, friable callus
10	MS_9	MS_0+ NAA(2.5mg/l)+Kn (1.25 mg/l)	+++	Creamy, green, profuse, friable callus
11	MS_{10}	MS_0 + NAA (2.5 mg/l) + Kn (1.75 mg/l)	+++	Creamy, green, profuse, friable callus
12	MS_{11}	MS_0 + NAA (5 mg/l) + Kn (2.5 mg/l)	+++	Creamy, green, profuse, friable callus
13	MS_{12}	MS_0 + BAP (1 mg/l) + NAA (2.5 mg/l)	+	Explant discoloration, callus initiation

On comparing the extraction efficiency of leaf and callus extracts, it was observed that callus extracts have more extractive value than leaf extracts which implies that the amount of secondary metabolites synthesized by the plant *in vitro* is more than *in vivo.* Callus extraction for phyto-constituents and its antimicrobial activity in different plants have been reported earlier by many workers (Arafa et al. 2016, Bhagya and Chandrashekar 2013, Jadhav et al. 2013, Singh 2011) but no such work has been reported previously on *Calotropis procera* and *C. gigantea.*

Table 2. Showing the percentage of initial extraction efficiency, final extraction efficiency (after subtracting the moisture content of leaf extracts).

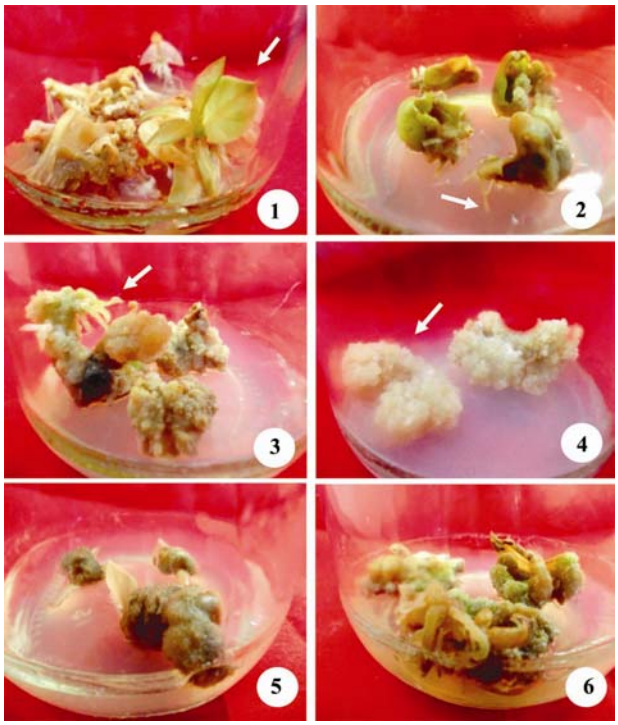
Sl. No.	Name of the plant	Type of extraction solvent	Initial weight of dried sample (g) *	Moisture Content *	Weight of extract (g) *	Weight of extract/100 g (% extraction efficiency) *	Final extraction efficiency (after subtracting moisture content) (%)*
1	C.P. (L)	50% Meth.	2	5.05 ± 0.03^{d}	0.0335 ± 3.34^{e}	1.674 ± 0.033^{d}	1.76 ± 0.041^{d}
2	C.G. (L)	50% Meth.	2	5.53 ± 0.04^{c}	0.0287 ± 5.14^{f}	1.445 ± 0.003^{e}	1.52 ± 0.027^{e}
3	C.P. (L)	90% Meth.	2	5.03 ± 0.04^{e}	0.0921 ± 2.54^{b}	4.605 ± 0.003^{b}	4.85 ± 0.033^{b}
4	C.G. (L)	90% Meth.	2	5.57 ± 0.03^{b}	0.0765 ± 3.04^{c}	3.834 ± 0.023^{c}	4.05 ± 0.003^{c}
5	C.P. (L)	Aq.	5	5.06 ± 0.05^{cd}	0.0561 ± 2.54^{d}	1.122 ± 0.002^{f}	1.18 ± 0.001^{f}
6	C.G. (L)	Aq.	5	5.58 ± 0.05^{a}	0.3154 ± 4.15^{a}	$\mathbf{6.306 \pm 0.004^{a}}$	$\mathbf{6.65 \pm 0.034^{a}}$
F (ANOVA)				184.082	453.71	220.91	304.89
LSD (P≤0.05)				0.0613	0.001	0.013	0.0368

*Each value is a mean of five replicates, ± = Standard deviation, the mean difference is significant at 0.05 level, values in columns followed by the same letter are not significantly different according to DMRT , ($p \leq 0.05$), least significant difference test.

Table 3. Showing the initial weight of dried callus, weight of extract and extraction efficiency of the callus extracts.

Sl. No.	Name of the media	Type of extraction solvent	Initial weight of dried callus (g) *	Moisture content (%)*	Weight of callus extract (g) *	Weight of callus extract/100 g (% extraction efficiency)*	Final extraction efficiency (After subtracting moisture content)*
1	C.P.(A)	50% Meth.	0.4237 ± 4.18^{g}	11.692 ± 3.53^{d}	0.0463 ± 2.77^{d}	10.92 ± 0.02^{c}	11.47 ± 0.04^{c}
2	C.P.(B)	"	0.4515 ± 4.03^{f}	10.295 ± 3.56^{e}	0.0565 ± 4.20^{d}	12.49 ± 0.02^{c}	13.65 ± 0.03^{c}
3	C.G.(A)	"	0.4513 ± 5.06^{f}	14.720 ± 0.01^{c}	0.0602 ± 1.58^{c}	13.33 ± 0.02^{c}	13.74 ± 0.04^{c}
4	C.G.(B)	"	0.7311 ± 4.15^{e}	23.368 ± 4.08^{b}	0.0613 ± 1.58^{c}	8.36 ± 0.01^{d}	9.26 ± 0.03^{d}
5	C.P.(A)	"	0.4235 ± 3.43^{g}	11.692 ± 5.06^{d}	0.0071 ± 2.54^{f}	1.65 ± 0.03^{e}	1.75 ± 0.03^{e}
6	C.P.(B)	"	0.4515 ± 3.53^{f}	10.295 ± 3.67^{e}	0.0985 ± 3.19^{b}	21.83 ± 0.02^{b}	23.86 ± 0.03^{b}
7	C.G.(A)	"	0.4513 ± 3.53^{f}	14.730 ± 0.03^{c}	0.0226 ± 4.18^{e}	5.05 ± 0.03^{d}	5.19 ± 0.02^{d}
8	C.G.(B)	"	0.7311 ± 3.53^{e}	23.362 ± 4.82^{b}	0.2896 ± 4.72^{a}	**39.64 ± 0.03^{a}**	**43.86 ± 0.03^{a}**
9	C.P.(A)	Aq.	2.8932 ± 0.07^{c}	11.692 ± 3.27^{d}	0.0167 ± 4.91^{ef}	0.584 ± 0.03^{e}	0.614 ± 0.03^{ef}
10	C.P.(B)	"	1.6030 ± 0.01^{d}	10.291 ± 0.06^{e}	0.0116 ± 1.94^{ef}	0.72 ± 0.01^{e}	0.77 ± 0.04^{e}
11	C.G.(A)	"	6.2870 ± 0.03^{b}	14.730 ± 0.02^{c}	0.0311 ± 2.58^{e}	0.49 ± 0.02^{ef}	0.511 ± 0.02^{ef}
12	C.G.(B)	"	7.2813 ± 4.54^{a}	23.378 ± 3.56^{a}	0.0270 ± 0.01^{e}	0.37 ± 0.01^{f}	0.40 ± 0.01^{f}
F (ANOVA)			448.87	763.78	950.44	1174.55	880.64
LSD ($p \leq 0.05$)			0.0032	0.0173	0.007	0.0309	0.0389

*Each value is a mean of five replicates, ± = Standard deviation, the mean difference is significant at 0.05 level, values in columns followed by the same letter are not significantly different according to DMRT, ($p \leq 0.05$), least significant difference test.



Figs 1-6. Showing callus proliferation of *Calotropis procera* and *C. gigantea* on different media: 1. Callus initiated from juvenile leaves of *Calotropis procera* on conc. A (MS_0 + NAA (2.5 mg/l) + Kn (2.5 mg/l). 2. Callus initiated from leaf segment of *Calotropis procera* on B conc. (MS_0 + NAA (5 mg/l) + Kn (2.5 mg/l) 3. Callus initiated from leaf segment of *Calotropis gigantea* on A concentration. 4. Callus initiated from leaf segment of *Calotropis gigantea* on B concentration. 5. Callus initiated from leaf segment of *Calotropis procera* on 2,4-D (6 mg/l). 6. Callus initiated from leaf segment of *Calotropis gigantea* on 2,4 -D (6 mg/l).

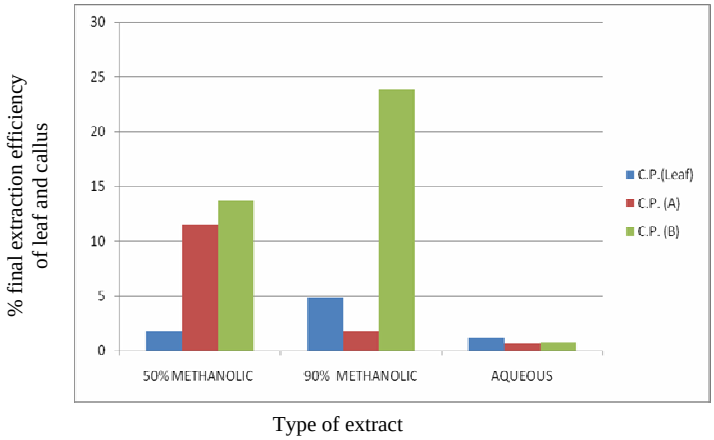


Fig. 7. Showing the comparison of extraction efficiency of different extracts of leaf and callus of *Calotropis procera*.

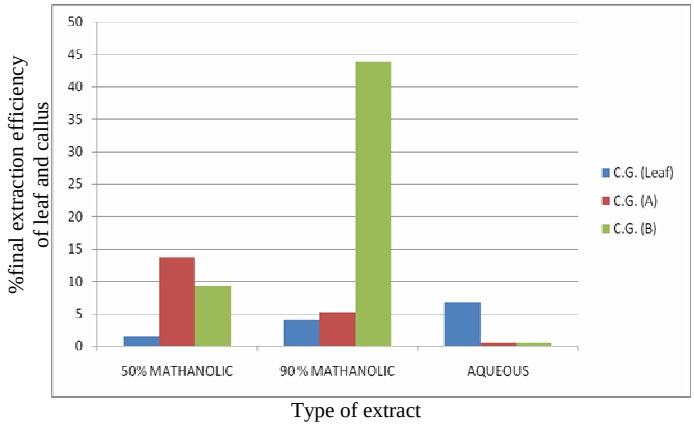


Fig. 8. Showing the comparison of extraction efficiency of different extracts of leaf and callus of *Calotropis gigantea*.

Callus cells are easily dissociable as they lack heavy cell wall depositions. In comparison, cell to cell compaction is very high in intact tissues and tissue dissociation requires treatment with a lot of mechanical and chemical tissue maceration methods. In our experimental observations, comparison between plant tissue and callus tissue showed a very significant increase in extraction efficiency. The type of tissue was found to be the governing factor of the above said parameters. The present study strengthens the fact that calculation and deduction of moisture content is important in extraction to enhance its extraction efficiency to a measurable extent.

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