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"Comparison of phytochemical extraction solvents and regeneration hormone combinations for *Andrographis paniculata*"

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Summary: Medicinal plants are of great use as they can be used to treat various diseases without side effects of the chemical drugs. *Andrographis paniculata* is one such plant possessing many health benefits like antioxidant, antibacterial, antitumor, antidiabetic, antithrombotic, anti-inflammatory and antiviral activities. In spite of all the known beneficial effects, there is no standardized protocol for extraction of bioactive components from this plant. So in the present study our main aim is to compare the different extraction solvents (acetone, chloroform, distilled water, ethyl acetate, hexane and methanol) for their efficiency in extracting *Andrographis Paniculata* plant's bioactive components. Further we also want to standardize the plant's regeneration protocol under laboratory controlled conditions using different hormone combinations. To best of our knowledge this is the first report which shows that the extraction of phytochemicals from the dried leaves of *Andrographis paniculata* is maximally obtained by using chloroform and distilled water as an extraction solvent and provides various hormone combinations for regeneration of this plant under laboratory controlled conditions.
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Introduction

Andrographis paniculata is a small annual herb with a strong bitter taste and belongs to the family Acanthacea. It is found in tropical and southeast Asia and is commonly called 'king of due to its extremely bitter taste (Kaskoos *et al.,* 2014). Other common names of this plant are "Hempedubumi" in Malaysia, "Chuan Xin Lian" in China, "Fa Thalai Chon" in Thailand, "Senshiren" in Japan and "Kalmegh" in India (Sareer et al.,, 2012) This plant is known to contain many bioactive compounds like steroids, phenols, terpenoids, alkaloids, saponins and flavonoids, primarily used by the plant for protection against pathogens (Cowan, 1999). Many medicinal properties accounts for these bioactive components for which it is used as an ayurvedic medicine since long times as these are less toxic and easy to consume (Table 1).

The major bioactive compound found in high amounts in *Andrographis paniculata* is Andrographolide, which is a diterpenoid lactone and found particularly in leaves. Primarily used by the plant for protection, these compounds possess many health benefits like antioxidant (Kamdem *et al.,* 2002), antibacterial (Singha *et al.,* 2003), antitumor, antidiabetic, antithrombotic, anti-inflammatory (Reddy *et al.,* 2005) and antiviral activities (Jayakumar, *et al.,* 2013). *Andrographis paniculata* is reported to be used in Traditional Chinese medicine (TCM) since ancient times to release body heat and toxins. It is used as an oral remedy against common cold, dysentery, fever, tonsillitis, diarrhoea, liver diseases, inflammation, herpes, influenza, sinusitis, antherosclerosis, insect and snake bites. It is also used for the treatment of leprosy, gonorrhea, scabies, boils, skin eruptions due to its blood purifying properties (Kumar, *et al.,* 2012).

This plant is normally grown in wild fields; however, it is difficult to meet the commercial demand due to limitations in the conventional production system for this plant (Martin, 2004). *In vitro* propagation is an alternative and efficient method to obtain the maximum yield of this valuable plant derived pharmaceuticals. Past research efforts for the regeneration of *Andrographis paniculata* have been taken by various groups using different plant parts like Karuppusamyet et al., in 2010 regenerated 30 day old seedlings and Bidari S.K. et al., in 2012 regenerated nodal explants of the plant by using different hormone combination.

However more research efforts are needed to provide a standard regeneration protocol.

Although the medicinal properties of this plant *Andrographis paniculata* are well known since ancient time, however, there is no standardized protocol for the extraction of active components from this plant. So in view of this the present study was undertaken to compare the efficiency of different extraction solvents in extracting the various phytochemicals present in the plant. We further also want to standardize its regeneration protocol under laboratory controlled conditions. We here provide the first report which shows that chloroform is the best extraction solvent for the extraction of the phytochemicals from the dried leaves of *Andrographis paniculata* and this plant can be easily regenerated using different hormone combinations under laboratory controlled conditions.

Experimental

Materials:

In this study, all the chemicals were provided by Hi-Media Co. including methanol, acetone, chloroform, ethyl acetate and hexane.

Collection of the plant sample and extract preparation

The plant has been taken from the Herbal Garden of Lovely Professional University. The plant leaves has been dried and crushed to make the fine paste. Extraction with different solvents like acetone, chloroform, distilled water, ethyl acetate, hexane and methanol have been done in soxhlet apparatus. Briefly, for every 200 ml of the each solvent, 25g of the crushed plant leaves powder was used for the soxhlet extraction. After extraction, the crude extracts were placed in water bath at 55˚C for evaporation and thus the crude extract of the plant for the each solvent was obtained.

Phytochemical Screening:-

Biochemical tests have been done to check the presence of different phytochemical such as alkaloids, flavonoids, saponins, steroids and tannins in the above mentioned *Andrographis paniculata* plant extract by the following procedure:

Test for alkaloids:- 10 mg of the each extract was taken and was dissolved in 2 ml of the Wagner's reagent for different extracts. After dissolving the both, the formation of reddish brown colored precipitates confirms the presence of alkaloids in the plant extract.

Test for flavinoids:- 10 mg of the each extract was taken and the few drops of diluted NaOH was to the each extract. The appearance of yellow color which disappears or become colorless on adding few drops of diluted H_2SO_4 confirms the presence of flavonoids in the plant extract.

Test for saponins:- 10 mg of the each extract was taken and each extract was diluted with 20 ml of distilled water. Then test tube was then shaken for 15 minutes by hand and the formation of foam on top of the test tube shows the presence of saponins in the plant extract.

Test for steroids:- 10 mg of the each extract was taken and the 1 ml of concentrated H_2SO_4 has been added to the each extract by the wall sides of the test tube. Appearance of dark reddish green color confirms the presence of steroids in the plant.

Test for tannins:- 10 mg of the each extract was taken and dissolved in 45% of the ethanol. Then the test tube was boiled for 5 minutes and 1 ml of 15% ferric chloride solution was added to each. The color appearance from greenish to black colour confirms the presence of tannins in the plant.

Antioxidant activity:-

Blois method (Blois, 1958) was used to determine the free radical scavenging activity of the plant extract by using DPPH (2,2-diphenyl-1-picrylhydrazyl). Briefly, 0.2 mM DPPH solution was made using methanol. Ascorbic acid was taken as the standard. 10 µg of the each extract was dissolved in 2ml of the mother solvent and 1 ml of prepared DPPH is added in all the tubes. The tubes were then kept in the dark for 60 minutes and the absorbance of all the samples was taken at 517 nm using spectrophotometer. The % inhibition was calculated by using the formula =

$$[(A_{control}-A_{extract}) / (A_{control})] * 100$$

Total phenolic content in the plant:-

To determine the total phenolic content of the plant, gallic acid was used as the standard. 20 µl of the each extract was taken and volume adjusted to 2 ml using parent solvent. 200 µl of FCR and 500 µl of 20 % Na_2CO_3 have been added to each tube. Then the reaction mixture was incubated for 1 hour at room temperature and the absorbance was measured at 760 nm.

Quantification of the flavonoids in the plant:-

For the quantification of the flavonoids, quercetin was used as the standard. Different concentrations of the quercetin (200 µl, 400 µl, 600 µl, 800 µl & 1 ml) have been used to make standard curve. For test samples 10 µl of each extract was dissolved in 100 µl of particular parent extract. The final volume for the each sample was adjusted to 2 ml by adding 100 µl of potassium acetate, 100 µl of aluminium chloride and rest distilled water. The samples were then incubated for 30 minutes at the room temperature and the O.D. was taken at 470 nm.

Plant regeneration:

Nodal parts of stem were used as explants for *in vitro* regeneration as described by Purkayastha J. *et al.*,2008.

Treatment of explants:

Explants were first washed under running tap water for 15 mins followed by washing with distilled water. They were then washed with tween 20 for 10 mins, followed by three washings with distilled water. Now the explants were soaked in 70% ethanol for 30 mins, followed by 3 washings with distilled water. They were then dipped in 0.01% $HgCl_2$ (prepared by dissolving 0.01g of $HgCl_2$powder in 100ml of distilled water) solution for 3 mins and washed with distilled water. Autoclaved test tubes were taken for inoculation of explants. Each test tube contained 20ml of MS media.

Preparation of media:

4.4g of MS media powder + 30 g sucrose (3% w/v sucrose) + 7g Agar powder (0.7%) was dissolved in distilled water by gentle heating and final volume was made 1000 ml. The pH of media was set 5.8 before autoclaving. The prepared media was autoclaved for 15 mins, at 15 psi and 121ºC temp. The micropipette tips, Petri plates used while inoculation were also autoclaved. Sterile blades were used for cutting the explants. About 1cm long sterilized nodal part cut from stem was inoculated in each test tube in upright position.

Hormone combinations used:

1.MS media
2. NAA 2 mg/l + BAP 0.05 mg/l
3. NAA 2 mg/l + BAP 1 mg/l
4. 2,4-D 2 mg/l + ZEATIN 0.05 mg/l
5. 2,4-D 2 mg/l + ZEATIN 1 mg/l
6. BAP 2 mg/l
7. BAP 2 mg/l + ZEATIN 2 mg/l
8. BAP 0.1 mg/l + ZEATIN 0.1 mg/l
9. BAP 1 mg/l

10 mg/ml stock solution was prepared by mixing 10 mg of hormone + 200 µl of NaOH + 800µl of distilled water and required concentration were added in the test tubes containing MS media. Inoculated explants were kept in culture room at 25ºC temperature and given a 16/8 hours photoperiod.

Statistical analysis

Experimental values are expressed as mean ± SEM. Comparison of mean values between various groups was performed by one way-analysis of variance (one way- ANOVA). P-value < 0.05 was considered to be significant.

RESULTS AND DISCUSSION

Extraction of active components from *Andrographis paniculata* plant using different solvents

The extracts from the dried leaves of the plant were made by using different solvents: acetone, chloroform, distilled water, ethyl acetate, hexane and methanol for three consecutive days each in soxhlet apparatus. The extracted aqueous extracts were kept in the water bath at 55˚C for evaporation of the solvent and the crude extract of the plant for the each solvent was obtained. After obtaining the extracts, the percentage yield of the extracts has been calculated (Table2). Our results show the maximum percent yield in obtained by using acetone as an extraction solvent (88.2%), followed by distilled water (36.76%), chloroform (27.16%), ethyl acetate (24.4%), methanol (21.32%) and hexane (17.88%). Hexane extraction results in minimum percent yield.

Phytochemical screening:-

Phytochemical screening tests have been performed to detect the presence of bioactive components in the plants. The results for the tests for the presence of saponins, tannins, alkaloids, steroids and flavonoids were as follows (Table 3).

- Test for saponins:- The extracts of all the solvents were diluted with the distilled water and the test tubes were shaken for 15 minutes by hand and the formation of the foam on the upper layer of the test tube showed the presence of saponins in it. All the extracts of the different solvents, except chloroform showed the positive result for the presence of saponins in the plant. The tests were performed in triplicates.
- Test for flavonoids:- All the extracts of the different solvents showed the positive result for the presence of flavonoids in the plant due to the appearance of the yellow colour in all.
- Test for alkaloids:- The extracts of the different solvents showed the appearance of reddish brown color in the test tubes which confirms the presence of alkaloids in the plant extracts obtained using different solvents.
- Test for steroids:- All the extracts of the different solvents showed the appearance of dark reddish green colour in the test tubes which confirms the presence of steroids in all the plant extracts.
- Test for tannins:- All the extracts of the different solvents, excluding chloroform showed the positive result for the presence of tannins in the plant due to the appearence of greenish black colour in the test tubes. The chloroform extract showed the negative result for the presence of tannins may be due to improper solubility.

Antioxidant activity

An antioxidant activity of all the plant extracts was observed using DPPH (2,2-diphenyl-1-picrylhydrazyl) and ascorbic acid as the standard. A standard curve was made and DPPH activity was observed by measuring OD at 517 nm. We observed that extracts from *Andrographis paniculata* plant formed using chloroform, distilled water and ethyl acetate shows the maximum antioxidant activity followed by acetone and methanol. Whereas hexane extract shows the least antioxidant activity (Table4).

Total flavonoid quantification in the plant:-

Total flavonoid concentration was quantified by using quercetin as the standard and measuring OD at 470 nm. Using hexane and methanol as an extraction solvent results in the maximum flavonoid extraction followed by distilled water, acetone and chloroform. When we use ethyl acetate as an extraction solvent, it results in the least extraction of flavonoids from *Andrographis paniculata* plant leaves (Table4).

Total phenolic content in the plant:-

Total phenolic content in the plant was estimated using gallic acid as a standard. The OD was taken at 760 nm. Maximum phenol concentration was observed when chloroform and distilled water was used as extraction solvent followed by acetone and ethyl acetate. When we use hexane and methanol as extraction solvent, it results in the least extraction of flavonoids from *Andrographis paniculata* plant leaves (Figure1 and Table4). This data directly correlates with antioxidant activity shown by these plant extracts (Figure1 and Table4).

Based on the above results we can say that chloroform and distilled water are the best extraction solvent for the extraction of phytochemicals from *Andrographis paniculata* plant and both works equally well.

Nodal explant regeneration of *Andrographis paniculata* in laboratory using different hormone combinations

Nodal parts of stem regenerated in different combinations of MS media (4.4g of MS media powder + 30 g sucrose (3% w/v sucrose) + 7g Agar powder (0.7%)). The prepared media was autoclaved for 15 mins at 15 psi and 121ºC temp. About 1cm long sterilized nodal part cut from stem was inoculated in upright position in test tube having autoclaved MS media prepared by dissolving 4.4g of MS media powder, 30 g sucrose (3% w/v sucrose), 7g Agar powder (0.7%) in 1000 ml distilled water. In each test different hormone concentration was added. Inoculated stems were incubated at 25 ºC in 16/8 hour photoperiod. Stems regeneration was seen on 2 mg/l BAP, 2mg/l BAP + 2 mg/l ZEATIN, 0.1 mg/l BAP + 0.1 mg/l ZEATIN, 2 mg/l 2,4-D + 0.5 mg/l ZEATIN hormone combinations added in MS media formulation (Figure 2). These results highlight that BAP and ZEATIN hormone combinations works best for *Andrographis paniculata* regeneration under laboratory controlled conditions.

Conclusion

In the present study we perform a direct comparison between the different extraction solvents for their efficiency in extracting the phytochemicals from Andrographis paniculata dried leaves. Further we also want to evaluate the effect of different hormone combinations on Andrographis paniculata plant regeneration potential. Our results clearly demonstrate that chloroform is the best extraction solvent as it results in maximum yield of flavonoids and phenol followed by distilled water and acetone. The antioxidant activity was also found to be maximum in chloroform followed by distilled water extract as demonstrated by DPPH method. These values are very well correlated with the total flavonoid and phenol content of these extracts. Further we have also demonstrated plant regeneration under laboratory controlled conditions and evaluated the effect of different hormone combinations. Our results clearly demonstrate that BAP and ZEATIN hormone combinations work very well resulting in regeneration of plant nodal areas.

Acknowledgments

The authors declare that no conflict of interest exists with the publication of this work.

Short Title: Phytochemical extraction and regeneration of Andrographis

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Figure 1:

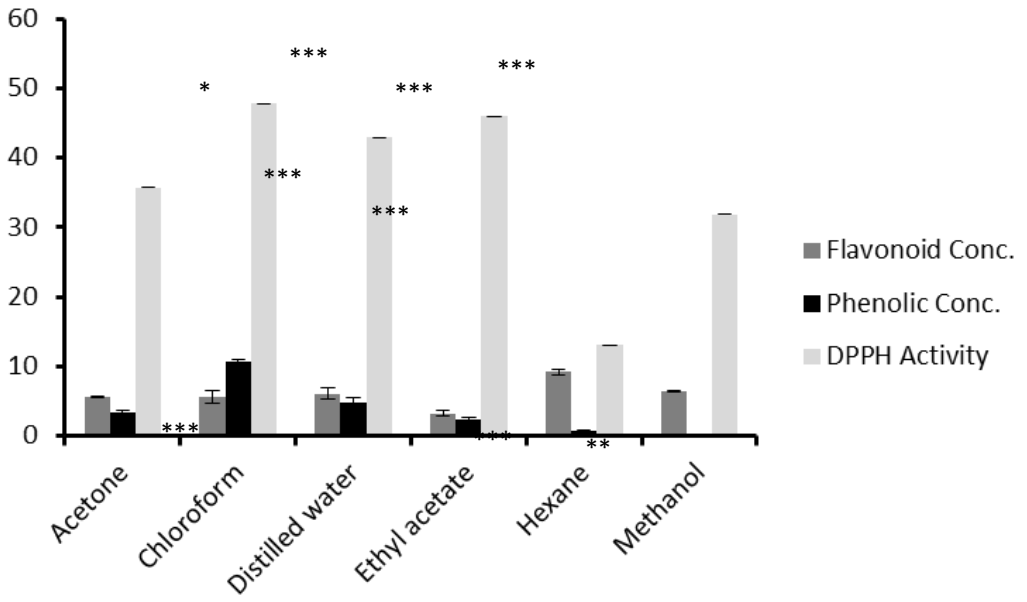

Figure 1: Comparison of flavonoid content, phenol content and DPPH activity from *Datura metel* plant extracts made with different solvents. Similar results were obtained in the three independent set of experiments. All the values were represented as mean±S.E.M. (n=3), ***p<0.001, **p<0.01 and *p<0.05, vs extraction with methanol.

Figure 2:

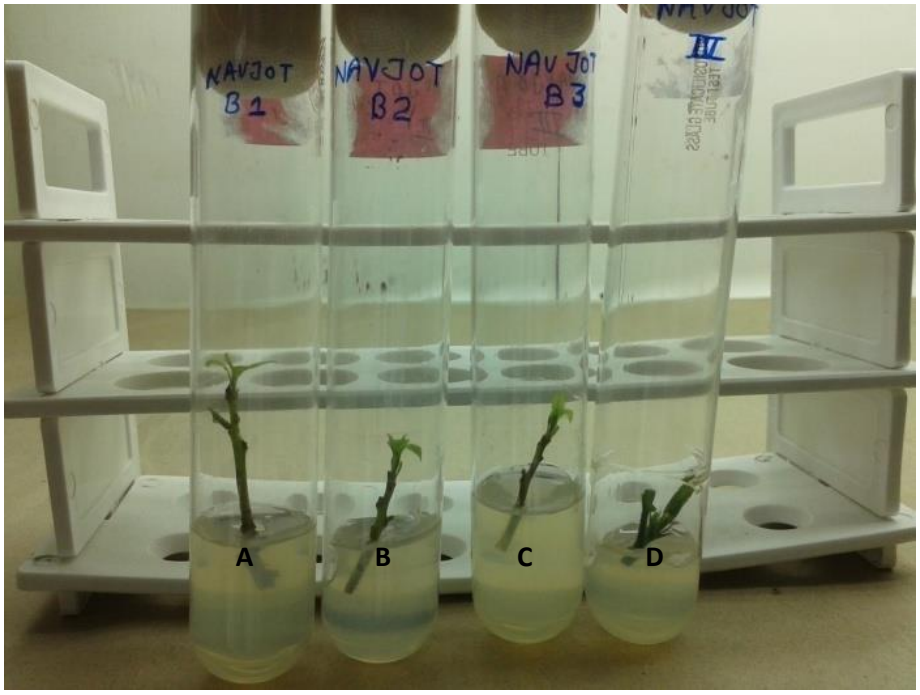

Figure 2: Nodal explant regeneration of *Andrographis paniculata* in laboratory using different hormone combinations. Nodal parts of stems of *Andrographis paniculata* inoculated in indifferent combinations of MS media + growth hormones and incubated at 25ºC and given 16/8 hour photoperiod. The picture in the above figure shows the nodal explants regenerated in A: 2 mg/l BAP, B: 2mg/l BAP + 2 mg/l ZEATIN, C: 0.1 mg/l BAP + 0.1 mg/l ZEATIN and D: 2 mg/l 2,4-D + 0.5 mg/l ZEATIN hormone combinations when added in MS media formulation.

Table 1: The basic structures and medicinal properties of various phytochemicals present in *Andrographis paniculata* plant

Phytochemical	Structure	Medicinal properties
Terpenoids	Isoprene units (consisting of five-carbons with two unsaturated bonds and a branched chain) in which the methyl groups are replaced by oxygen atoms	Anticarcinogenic, antiulcer, antimicrobial, antioxidant, antimalarial and antidiuretic (Nayak, 2012)
Phenols	A aromatic ring with a hydroxyl group	Antimicrobial, antioxidant (Nice. 2009), (Sahoo, 2012)
Flavonoids	Phenolic aromatic molecules having a carbonyl group	Anti-inflammatory, antithrombotic, antioxidant, antiviral (Cowan, 1999)
Saponins	One or more hydrophilic glycoside units with lipophilic triterpene derivative	Antidiarrheal, anticancer, and antihelmintic (Sahoo, 2012)
Tannins	One or more hydrophilic glycoside units with lipophilic triterpene derivative	Antioxidant, antiperoxidative, antimicrobial, antimutagenic, antidiabetic and antiviral (Sahoo, 2012)

Table 2: Percentage yield of the extracts made from *Andrographis paniculata* plant using different extraction solvents (Weight of extract obtained / Weight of dry powder used) * 100

EXTRACT NAME	PERCENT YIELD OF EXTRACT	COLOUR OF EXTRACT
Acetone	88.2%	Dark green
Chloroform	27.16%	Light green
Distilled water	36.76%	Dark brown
Ethyl acetate	24.4%	Dark green
Hexane	17.88%	Light green
Methanol	21.32%	Dark green

Table 3:- Results for the photochemical screening in different extraction solvents from *Datura metel* plant

TEST	METHANOL	EHTYL ACETATE	CHLOROFORM	DISTILLED WATER	ACETONE	CONTROL
ALKALOIDS	++	+	+++	+	+	-
FLAVONOIDS	++	+	+	+++	++	-
SAPONINS	-	++	+	+++	++	-
STEROIDS	++	++	+	+	++	-
TANNINS	++	+	-	++	++	-

+ = presence; - = absence

Table 4: Comparison of flavonoid content, phenol content and DPPH activity from *Andrographis paniculata* plant extracts made with different solvents

EXTARCT	FLAVONOID CONC. (mg/g)	PHENOL CONC. (mg/g)	%INHIBITION ACTIVITY OF DPPH (%)
ACETONE	5.6 ± 0.2	3.4 ± 0.2	35.75 ± 0.03
CHLOROFORM	5.6 ± 1	10.6 ± 0.3	47.75 ± 0.008
DISTILLED WATER	6.1 ± 0.9	4.7 ± 0.7 ***	42.9 ± 0.009 * ***
ETHYL ACETATE	3.2 ± 0.4	2.45 ± 0.3***	45.95 ± 0.0015**
HEXANE	9.2 ± 0.4	0.8 ± 0.01 ***	13.1 ± 0.01 ***
METHANOL	6.5 ± 0.1	0.22 ± 0.06**	31.85 ± 0.0025

Similar results were obtained in the three independent set of experiments. All the values were represented as mean±S.E.M. (n=3), ***p<0.001, **p<0.01 and *p<0.05, vs extraction with methanol.

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