ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 05, Issue, 08, pp. 5209-5214, August, 2015

International Journal of
DEVELOPMENT RESEARCH

Full Length Research Article

PHYTOCHEMICAL SCREENING AND ANTIOXIDANT ACTIVITY OF LEAVES AND CELL SUSPENSION CULTURE FROM SEED DERIVED CALLUS OF *SPINACIA OLERACAE*

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ARTICLE INFO

Article History:
Received 22nd May, 2015
Received in revised form
13th June, 2015
Accepted 28th July, 2015
Published online 31th August, 2015

Key words:

Spinacia oleracae,
Phytochemical,
Total phenolic content,
Antioxidant,
Seed derived callus,
Cell suspension biosynthetic pathways

ABSTRACT

Objective: To screen the phytochemical constituents as well as to assess the total phenolic content and antioxidant potential of plant extract(leaves) and cell suspension culture extract from the seed derived callus of *Spinacia oleracae*.

Methods: Establishment of callus was carried out by use of MS medium supplemented with growth hormones, BAP and IAA with some modifications in the standardized method. Plant extract was subjected to the phytochemical screening as reported methods. Folin–Ciocalteu reagent was used to estimate total phenolic content and the antioxidant potential was determined by DPPH radical scavenging assay.

Results: Phytochemical screening of extract showed presence of major classes of phytochemicals such as Alkaloids phenols, tannins, flavonoids, terpenoid, carbohydrates, glycosides, reducing sugars and proteins. It was found that the seed derived callus formation was maximum after 28th day and satisfactory establishment of cell suspension culture was observed after the 25th day of inoculation. An antioxidant potential of cell culture extract was found to be 75.78 %where the value was 68.47% for leaf extract.Total phenolic content of plant extract (leaves) and cell suspension culture extract was found to be 4.85mg GAE/g and 3.96 mg GAE/g respectively.

Conclusion: The result revealed that the leaf extract showed higher total phenolic conent as compare to cell culture extract However,an antioxidant potential of leaf extract was found to be lower than the cell culture extract. The possible reason for increase in anioxidant potential of cell culture extract may be due to stimulation of biosynthetic pathways of primary and secondary metabolites which may be responsible for antioxidant activity. Thus the present study will be useful for application of cell culture derived from spinach seeds , as a promising alternative to the whole plant for the large scale production of plant phenolics.

INTRODUCTION

Vegetables are the rich source of bioactive substances which supports the defense mechanism against various diseases. It is well known that *Spinaciaoleracea* (Spinach) is highly enriched with important secondary metabolites having bioactive constituents like phenolic acids, tannins, flavonoids, carbohydrates, glycosides, steroids, terpenoids, fats and lignins (Ergene *et al.* ,2006). These compounds are traditionally used in medicines for numerous therapeutic effects like laxative, diuretic, carminative and shown to possess anti-inflammatory, antioxidants, anti-mutagenic potential, anti-neoplastic effects as well as chemo-preventive activites (Kirtikar and Basu, 2005). The imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage which can cause several diseases. Recently, much attention has been focused on the phenolic content due to their antioxidant properties and different preventive role against the diseases associated with oxidative stress (Riedel *et al*., 2010; Scalbert and Williamson, 2000). As the invitro culture have potential to form secondary metabolites and to exhibit bioactivity comparable to original plant, the cell suspension culture may proved to be a useful tool for producing active secondary metabolites under controlled conditions (Ahuja *et al.,* 1986; Parsaeimehr *et al.,* 2010; Ravishankar and Ramachandra Rao, 2000).

Jameel and his team (1992) has studied the effect of 2,4-D and kinetin in combination on mature seeds derived callus of *S. oleracea* and scanty references are found on the seed

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derived callus formation of *S. oleracea* by using IAA and BAP. In view of the reputed efficacies of *Spinaciaoleracea,* this study aims at phytochemical screening and assessing the antioxidant (radical scavenging) potential and total phenolic content of the leaves and cell suspension culture.

MATERIALS AND METHODS

Collection of Plant Material

The plant material (leaves of *Spinaciaoleracea)* was collected from the agricultural field of Nashik, India. The sample was collected in plastic zip lock bags and brought to the laboratory where it was twicely washed with tap water followed by double distilled water then it was shed dried and powdered for further extraction. 5 gm. of powder was soaked in 50mL methanol and incubated on orbital shaker for 2 hours at room temperature. After the incubation period is over, the mixture was filtered by use of Whatman^{TM} filter paper No.1.Then the extract was concentrated to dryness in rotary evaporator and stored at 4ºC for further phytochemical analysis by use of standard method (Dharmendra Singh *et al*., 2012; Anjali Soni and Sheetal Sosa, 2013; Pascaline *et al.,*2011)

Seed Sterilization

Seeds of Spinaciaoleracea were obtained from local seed market, Nashik., India. Seeds were washed with tap water for 1hr followed by washing with 70% Ethanol (V/V) for 2 min and 5% Calcium hypochlorite for 30 min. Further the seeds were rinsed with sterile distilled water for 6 times and suspended for 24 hr. to soften the seed coat. On the next day, seeds were rinsed with sterile distilled water and small incisions were made into the seed coats with a scalpel to facilitate the callus induction (Jameel *et al.,* 1992).

Callus Culture

Callus culture was established by standardized method of Jameel *et al.,* 1992 with some modifications. Surface sterilized Seeds were inoculated on full strength MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose, 0.8% agar and various concentrations of Indole-3-acetic acid (IAA) and 6-Benzylaminopurine (BAP) ranging from 0.1,0.5,1.0,1.5,2.0 mg/L in combination. The pH of medium was adjusted to 5.6-5.7and then autoclaved at 121ºC for 15 mins. The culture was incubated at 24±2 ºC with 8 h dark and 16 h light period for 4-5 weeks. The combine effect of growth hormones in culture medium was screened for proper callus induction. Subculturing was carried out to maintain the callus for further use.

Suspension Culture

2 gm callus material was collected in the active growth phase(after the 15th day of subculture) it is then placed in 250 mL flask containing 100 mL liquid M.S. basal culture medium supplemented with 3% sucrose and 0.5mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D). The pH of medium was adjusted to 5.8 prior to autoclaving. Flasks were kept on rotary shaker at 120 rpm maintained at 27±2ºC for 15 days with photoperiod 16h light/ 8h dark (Robles-epeda *et al*., 2009)

Preparation of Plant Extract

5 gm of powdered leaves were placed in conical flask .50 mL of methanol was added and plugged with cotton. The powder material was extracted with methanol for 24 h at room temperature with continuous stirring. After 24 hours the filtrate was collected using Whatman^{TM} filter paper No.1.Then it was evaporated to dryness in rotary evaporator to get crude extract. The residue obtained was stored in airtight bottles in a refrigerator for further phytochemical analysis by use of standard method (Anjali Soni and Sheetal Sosa, 2013; Mohd Hafiz Ibrahim *et al*., 2011).

Preparation of Cell Suspension Culture Extract

The cell suspension culture was centrifuged at 3000 rpm for 10min. Then 100 mL supernatant was extracted with 30mL methanol and left for 2h on the orbital shaker at 120 rpm. The methanolic extract was evaporated to dryness by using a rotary evaporator at 45 ^{0}C for 40 min. The crude extract was stored at 4^{0}C and used for determination of total phenolic content (Ilieva *et al.,* 2014).

Preliminary Phytochemical Screening

Sample (1mLMethanolic extract for each test) of plant material (leaves) was subjected to chemical analysis for detection of different phyto-constituents using standard methods.

Test for Alkaloids

Sample was warmed with 2% H_2SO_4 for two minutes then it is filtered .Creamy-white colored precipitation by addition of few drops of Mayer's reagent indicates presence of alkaloids (Mohd Yousuf Malla *et al*., 2013).

Test for Tannins

2 drops of ferric chloride reagent (5% $FeCl_3$ in 90 % ethanol) were added to the sample. A bluish black color indicates the presence of Tannins (Farah K. Abdul-Wahab and Thukaa Z. Abdul Jalil, 2012).

Test for Saponins

Sample was mixed with 5mL distilled water and shaken vigorously. Formation of stable foam by addition of few drops of olive oil indicates presence of saponins (Anjali Soni and Sheetal Sosa, 2013).

Test for Flavonoids

Shinoda test: Crude extract was mixed with few fragments of magnesium ribbons and conc. hydrochloric acid was added drop wise. Orange coloration indicates presence of flavonoids (Usman *et al.,* 2009).

Test for Phenols

Sample was dissolved in 5mL distilled water followed by addition of 3mL of 10% lead acetate solution. A bulky white

precipitate indicates the presence of phenolics (Farah K. and Thukaa Jalil, 2012).

Test for Terpenoids

1 mL of acetic anhydride was added in sample followed by the addition of conc. H_2SO_4. A change in colour from pink to violet showed the presence of terpenoids (Sofowora, 1993; Usman *et al*., 2009).

Test for Steroids

2mL of acetic anhydride was added in 0.5 mL of sample, along with 2mL concentrated H_2SO_4. The colour changed from violet to blue or green indicates presence of steroids (Anjali Soni and Sheetal Sosa, 2013).

Test for Carbohydrates

Few drops of Molisch's reagent was added to sample this was then followed by addition of 1 mL of conc. H_2SO_4 by the side of the test tube. The mixture was then allowed to stand for two minutes and then diluted with 5 mL of distilled water. Formation of a red or dull violet colour at the interphase of the two layers was a positive test (Usman *et al*., 2009; Sofowora, 1993).

Test for Glycosides

5mL of diluted sulphuric acid was added to the sample and boiled for 15 minutes in a water bath. After cooling, it was neutralized with 20% potassium hydroxides solution. A mixture of 10mL of equal parts of Fehling's solution A and B were added and boiled for five minutes. Formation of dense red precipitate indicates the presence of glycosides (Dharmendra Sing *et al*., 2012).

Test for Reducing Sugar

Fehling's solution A and B were added to the Sample in equal amount. Mixture is shaked well and placed in boiling water for few minutes. Formation of brick red precipitation indicates presence of reducing sugar (Dharmendra Sing *et al*., 2012).

Test for Proteins

Biuret Test – Sample was treated with 10% sodium hydroxide solution and two drops of 0.1% copper sulphatesolution formation of violet/pink color indicates presence of proteins (Satheesh Kumar Bhandary *et al*., 2012).

Qualitative Test for phenolic content of cell culture extract

1mL of ferric chloride reagent (5% $FeCl_3$ in 90 % ethanol) was added to 1mLcell culture extract prepared in distill water and shaked well. Change in colourfrom orange to black indicates presence of phenolics. Gallic acid was used as standard.

Qualitative Analysis of phenolic constituents in the cell culture extract of *S.oleracae* by Thin Layer Chromatography (TLC)

The phenolic constituent in the cell culture extract were analysed by use of thin layer chromatography (TLC). About 100 µL of extract was loaded on TLC plate (Merck, 10 x10cm^2). The plate was developed in Chloroform: Methanol (9:1) for separating various constituents of the extract. 5% Anisaldehyde was sprayed on plate and incubated for15-20 minutes at 40^{0}C. Dark blue spot was noted for the Rf value. Gallic acid was used as positive control.

Determination of Antioxidant Activity DPPH Radical Scavenging Assay

The hydrogen atom or electron donation abilities of the plant extract, cell culture extract and gallic acid (standard) were measured from the bleaching of a purple-colored methanolic solution of DPPH. This spectrophotometric assay uses the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a reagent (Amarowicz *et al*., 2004). An aliquot of the standard and samples (250 µg/mL) each was added to 1mL of 0.3 mM /lit DPPH reagent (Sigma-Aldrich).

The mixture was shaken vigorously and then incubated in dark at 37ºC for 30 minutes. UV-Vis spectrophotometer (Systronics-572, India) was used to monitor the decrease in absorbance at 517 nm where, DPPH reagent with 1mL methanol was taken as control. The radical-scavenging activities of samples were expressed as percentage inhibition of DPPH by using the formula:

% free radical scavenging activity = $[A_c\text{-}A_s/A_c] \times 100$

Where A_c is absorbance of control and A_s is absorbance of sample.

Estimation of total phenolic

The total phenolic content of plant material extract and cell suspension culture extract was determined by using method of Mcdonald et al. (2001). Calibration curve was prepared by mixing methanolic solution of gallic acid (1mL; 0.1-0.6mg/mL) with 5mL Folin- Ciocalteu reagent (diluted 10 fold) and sodium carbonate (4mL; 0.7M).

Then the reaction mixture was allowed to incubate for 30minutes at room temperature and absorbance was measured by using double beam spectrophotometer (Systronics -572, India) at 765nm. Similarly, total phenolic content of samples was determined by using 1mL extract (100mg/mL). Then, the total phenolic content was calculated as gallic acid equivalent (GAE) by the following equation:

$T=C.V/M$

Where,

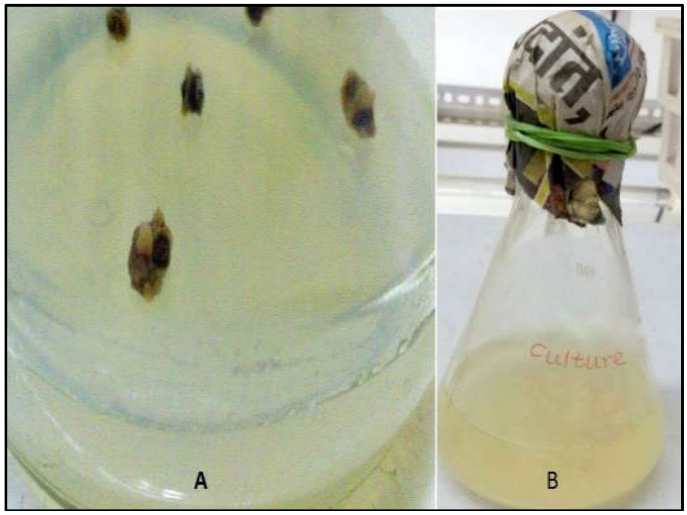
T= total phenolic content in mg/g of the extracts as GAE;

C= concentration of gallic acid established from the calibration curve in mg/mL;

V= volume of the extract solution in mL and M= weight of the extract in gm.

RESULTS AND DISCUSSION

There is a strong influence of type and concentrations of the auxins and cytokinins either used alone or in combination on the growth of callus as well as secondary metabolite production in plant tissue culture (Khemani, LD). So, the present study has been carried out to study the callus formation by using IAA and BAP. It was observed that there is maximum callus formation in MS medium supplemented with 1.5mg/L IAA and 0.5mg /L BAP only after 28th day. (Fig. 1A) Cell suspension culture was successfully established from the seed derived callus of *S. oleracea* after 25th day of inoculation. (Fig. 1B)

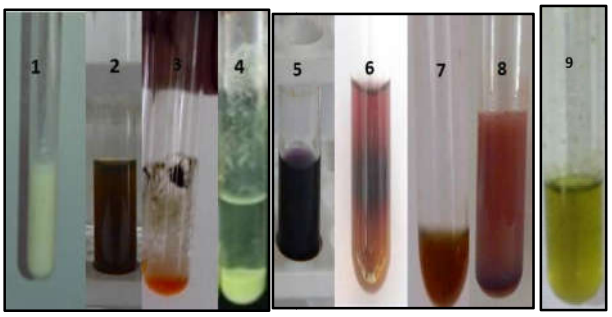


[A. Callus induced from mature seeds of *S.oleracae* B. Cell suspension culture]

Fig. 1. Callus induced from mature seeds of *S.oleracae* and established Cell suspension culture

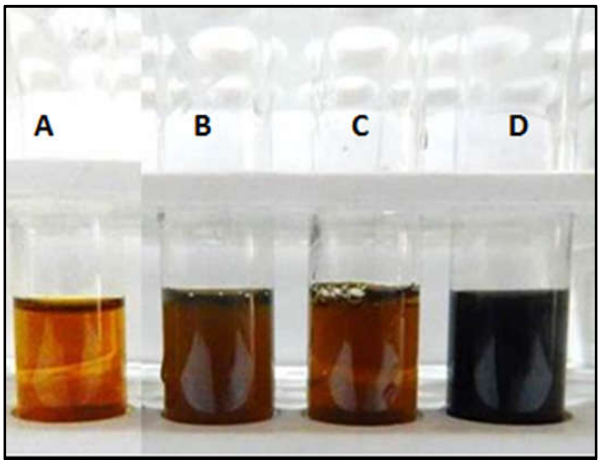
Phytochemical Analysis

Preliminary phytochemical analysis of leaf extract shows presence of major classes of phytochemicals such as Alkaloids phenols, tannins, flavonoids, terpenoid, carbohydrates, glycosides, reducing sugars and proteins Test for steroids and Saponins was found to be negligible in the leaf extract (Table 1) and (Fig.2). Qualitative test of phenolics for the cell suspension culture extract by use of FeCl3 reagent shows presence of phenolic content (Fig.3)



1. Alkaloids; 2. Tannins; 3.Flavonoids; 4.Phenols; 5.Terpenoids; 6. Carbohydrates; 7. Glycosides; 8.Reducing sugar; 9.Saponin

Figure 2. Phytochemical Screening of leaf extract (*Spinacia oleracea)*



[A. Blank B. Plant Extract C. Cell Culture Extract D. Standard]

Fig. 3. Qualitative test for Phenolics in Cell culture extract

Qualitative Analysis of Phenolic Constituents in Cell Culture Extract by Thin Layer Chromatography

The TLC plate shows three prominent spots among which one is confirmed as phenolics (Rf value 0.39) which corresponds to the Rf value (0.41) of standard (gallic acid). The possible reason for presence of other spots may be due to other phenolic constituents in the sample (Fig. 4).

Table 1. Preliminary phytochemical evaluation of *Spinacia oleracea*

Sr. No.	Active constituents	Tests	Result
1	Alkaloids	Mayer's Test (Mohd Yousuf Malla *et al*., 2013)	+
2	Tannins	FeCl3 test (Farah K. and Thukaa, 2012)	+
3	Saponins	Froth test .(Anjali Soni and Sheetal sosa, 2013)	-
4	Flavonoids	Shinoda test (Usman H *et al*., 2009)	+
5	Phenols	Lead acetate test (Farah K. and Thukaa Z., 2012)	+
6	Terpenoids	(Sofowora, 1993; Usman H *et al*., 2009).	+
7	Steroids	Acetic anhydride and H2SO4 reagent .(Anjali Soni and Sheetal sosa, 2013)	-
8	Carbohydrates	Molisch's test (Usman *et al*., 2009; Sofowora, 1993)	+
9	Glycosides	Fehling's reagent .(Dharmendra Sing *et al*., 2012)	+
10	Reducing sugars	Fehling's reagent .(Dharmendra Sing *et al*., 2012)	+
11	Proteins	Biuret Test (Satheesh Kumar Bhandary *et al*., 2012)	+

(+ positive test ; - negative test)

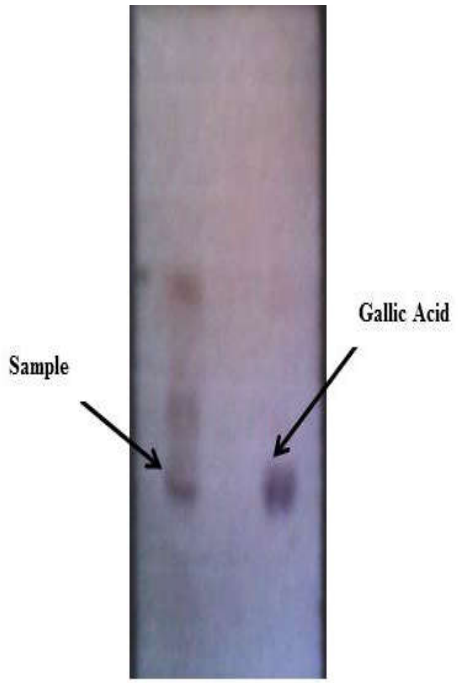
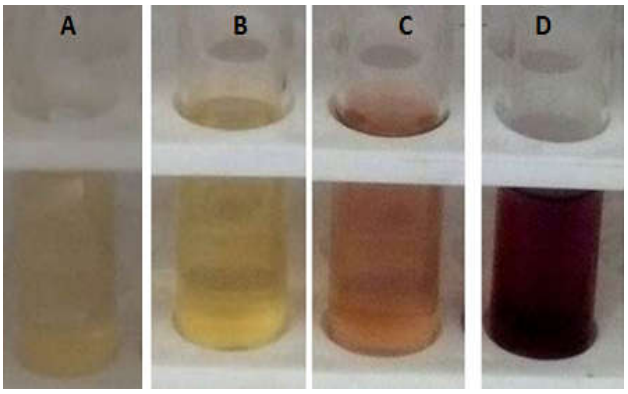


Fig. 4. Qualitative Analysis of phenolic constituents by TLC

Antioxidant activity

DPPH-Radical Scavenging ant Total Antioxidant Activity (TAC)

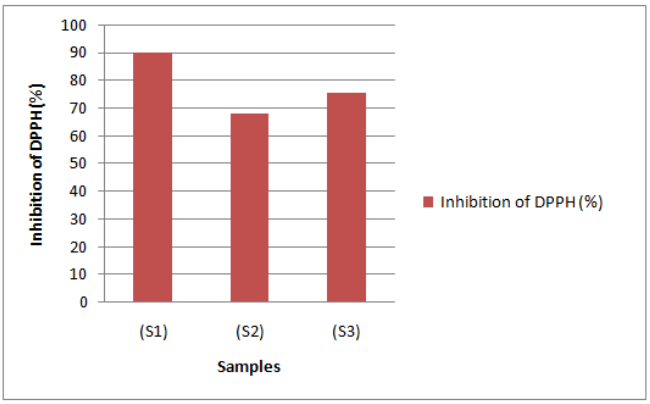
The DPPH assay has been largely used as a quick, reliable and reproducible parameter tosearch for the in vitro antioxidant activity of pure compounds as well as plant extracts (Burda and Oleszek, 2001; Ara and Nur, 2009). The radical scavenging activity of the extract was observed from the decrease in absorbance of the DPPH with the leaf extract ,cell culture extract and compared with standard at 517nm (Fig.5).



A .Gallic acid (standard) B. Cell culture extract C. Leaf extract D. Control

Fig. 5. Antioxidant activity of by DPPH Assay

This manifested in the rapid discolouration of the purple DPPH to light yellow, suggesting that the radical scavenging activity of methanol extract of samples was due to its proton donating ability. It was found that methanolic extract of plant and cell culture exhibited antioxidant activity by 68.47% and 75.78% respectively as compare to gallic acid which shows antioxidant potential of 90.06%. The possible reason for increse in anioxidant potential of cell culture extract may be due to stimulation of biosynthetic pathways of primary and secondary metabolites responsible for antioxidant activity. (Graph.1)



S1 .Gallic acid standard S2.Leaf Extract S3.Cell culture extract

Graph 1. Antioxidant Activity of leaf Extract and Cell culture extract by DPPH assay

Total Phenolic Content

Total phenolic content of leaf extract was found to be4.85 mg GAE/g (Gallic acid equivalent) and 3.96 mg GAE/g for cell suspension culture extract. It is reported that the phenolic content from extracts has a correlation with radical scavenging activity (Li *et al.,* 2009; Sim *et al*., 2010). This is because the polyphenolics have high redox potentials which allow them to act as reducing agent, hydrogen donors and singlet oxygen quenchers (Kahkonen *et al*, 1999).

Acknowledgement

The authors are highly thankful to Department of Biotechnology, K.T.H.M College, Nashik, India for providing the laboratorial facilities and valuable assistance for the completion of the research work.

REFERENCES

Ahuja, M., Evens, D., Sharp, W and. Am-mirato P., 1986. *Handbook of Plant Cell Culture, Macmillan, New York*: 626-651

Amarowicz, R., Pegg, R., Moghaddam, P., Barl, B., Weil, J., 2004. Free-radical scavenging capacity and antioxidant activity of selected plant species from Canadian prairies. *Food Chem.*, 84:551-562

Ara, N., Nur, H., 2009. In vitro antioxidant activity of methanolic leaves and flowers extracts of Lippiaalba. Res. *J. Med. Medical Sci*.,4(1): 107-110

Burda, S. and Oleszek, W., 2001.Antioxidant and Antiradical Activities of Flavonoids. *J. Agric. Food Chem.,* 49: 2774-2779

Ergene A., Guler P., Tan S., Mirici S., Hamzaoglu E., Duran A., 2006. Antimicrobial and antifungal activity of Heracleumsphondyliumsubsp. artivinense, *African Journal of Biotechnology.*, 5(11): 1087–1089

Farah K., Abdul-Wahab and Thukaa Z. Abdul Jalil, 2012. Study of Iraqi Spinach Leaves Phytochemical and

Protective Effects Against methotrexate-Induced hepatotoxicity in rats, Iraqi J Pharm Sci., 21(2):8-17

Hafiz, M., Hawa, Z., Rahmat, A. and Zaharah A, 2011. Effects of Nitrogen Fertilization on Synthesis of Primary and Secondary Metabolites in Three Varieties of Kacip Fatimah (Labisia Pumila Blume). *International journal of Molecular sciences* 12, 5238-5254

Ilieva1, M., Pavlov1, A., Kovatcheva, E., Michneva, M., 1995 Growth and Phenolics Production of Cell Suspension Culture of Lavandula Vera. Biotechnology and Biotechnological equipment MM.,9(4):69-71

Jameel, M., Al-Khayri., Feng, H., Huang. Teddy E., Morelock and Tahani A. Busharar, 1992. In vitro plant regener ation of spinach from mature seed derived callus. In Vitro cell. *Dev. Biol.*, 28: 64-66

Kahkonen , M., Hopia, A., Vuorela, H., Rauha, J., Pihlaja, K., Kulaja, T., Heinonen, M., 1999.Antioxidant activity of plants extracts containing phenolics compounds. *J. Agric. Food Chem.*, 47:3954-3962

Kirtikar, K., Basu, B., 2005. Indian Medicinal plants *International Book Distributors Deharadun*; 8: 2078-2079

Li, X., Wu, X., Huang, L., 2009. Correlation between antioxidant activities and phenolic contents of radix Angelicaesinensis (Danggui). *Molecules*: 5349-5361

Parsaeimehr, A., Sargsyan, E. and Javidnia, K., 2010. Comparative Study of the Antibacterial, Antifungal and Anti-oxidant Activity and Total Content of Phenolic Compounds of Cell Cultures and Wild Plants of Three Endemic Species of Ephedra. *Molecules.,* 15(3):1668-1678

Pascaline, J., Charles, M., Lukhoba C, 2011. Phytochemical constituents of some medicinal plants used by the Nandis of South Nandi district Kenya. *Journal of Animal & Plant Sciences*., 9(3): 1201- 1210

Ravishankar, G and Ramachandra S., 2000. Biotechnological production of phyto pharmaceuticals. *Journal of Biochemistry Molecular Biology and Biophysics*., 4, 73–102

Riedel, H., Cai, Z., Kutuk, O, Smetanska, I, 2010. Obtaining phenolic acids from cell cultures of various Artemisia species. *Afr. J. Biotechnol*. 9(51):8805-8809

Robles-Zepeda, R., Jimenez-Estrada, M., Navarro-Ocana A., Seed-Villegas I., Brunner I., Ruiz-Bustos, E., 2009. Secondary metabolites induction in mammillaria Huitzilopochtli (cactaceae) and evaluation of the fungicidal activity, *African journal of Biotechnology.,* 8(16): 3874-3878

Scalbert, A, Williamson, G, 2000. Dietary intake and bioavailability of polyphenols. *J. Nutr*., 130(8):2073-2085

Sim, K., Sri Nurestri, A., Norhanom, A, 2010. Phenolics content and antioxidant activity of Pereskiagrandifolia Haw. (Cactaceae) extracts. *Pharmacog. Magazine*, 6(23): 248-254

Singh, D., Singh, P., Gupta, A., Solanki, S., Sharma, E., Nema, R., 2012. Qualitative Estimation of the Presence of Bioactive Compound in Centella Asiatica: An important Medicinal Plant, *International Journal of Life Science and Medical Science.,* 2(1): 5-7

Smetanska, I., 2008. Production of secondary metabolites using plant cell cultures. *Advance in Biochemistry and Engineering Biotechnology.*, 111, 187–228

Snyder, W. and Hansen, H.,1940. The species concept in Fusarium. *Amer. J. Bot*. 27:64-67

Soni, A. and Sosa S., 2013. Phytochemical Analysis and free radical scavenging potential of herbal and medicinal plant extracts. *Journal of pharmacognosy and phytochemistry.*, 2(4):22-29

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