International Journal of Phytomedicine 6 (2014) 377-383
http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

In vitro culture of the moss *Hyophillanymaniana* (Fleish.)Menzel and its phytochemical screening

Rashmi Mishra[1], Vijay Kant Pandey[1], Ramesh Chandra[1]*

*Corresponding author:

Ramesh Chandra

[1]Department of Bioengineering, Birla Institute of Technology, Mesra, Ranchi, India

Abstract

Bryophytes are the second largest group of land plants after angiosperm and are considered as a remarkable reservoir of many new, natural products or secondary metabolites, which have shown interesting biological activity. The full economical potential of bryophytes is not completely explored. The present study aims to establish an efficient protocol for in vitro culture of one of the species of moss Hyophillanymaniana(Fleish.) Menzeland to perform phytochemical screening of this moss. Media content as well as light and temperature were varied to get the optimum condition for spore germination. Maximum number of spore germinated in 1/4 strength MS containing 1.5% sucrose and 2% of NaOCl was found to be the best for surface disinfection of sporophyte. Temperature range of 22±1 C and pH 6 along with light intensity of 2000-4000 lux with alternate day and night condition of (16/8 hours) was found suitable for the growth of the plant. For phyotochemical screening the methanolicethanolic and ethyl acetate extract of the moss prepared. Ethanolic extract showed the presence ofalkaloids, glycosides, steroids and terpenoids.

Keywords: *Hyophillanymaniana* (Fleish.) Menzel, *in vitro* culture, sodium hypochlorite, MS medium.

Introduction

Bryophytes are the second largest group of higher plants (comprising hornworts, liverworts and mosses) after lower plants, with estimated about ([1, 2, 3] 20,000to 25,000 species worldwide. They are the most exotic and captivating species on earth with a unique combination of distinguishing characteristics. They are among oldest land plant devoid of true vascular system and like all land plants (embryophytes), show 'alternation of generations'. Bryophytes especially mosses are the source of many biologically active novel compounds pertaining to pharmaceutical uses [4, 5, 6, 7].They are considered as a 'remarkable reservoir' of novel natural products or secondary metabolites, which have shown interesting biological activity which could be used in medicine. They possess compounds such as alkaloids (clavatoxine, clavatine, nicotine, lycopodine) polyphenolic acids (dihydrocaffeic) and flavonoids (apigenin, triterpenes, etc.) but only few of the species have yet been thoroughly studied.

Although, mosses are the source of many biologically active compounds[8, 9] only few of the species have yet been explored so far and very less attention has been given in context to *in vitro* culture. Despite of easy cultivation and establishment of axenic culture of mosses in laboratory, there are many discrepancies in the response of different moss species to the same treatment under *in vitro* culture [10]. Therefore, only a small amount work has been done in culturing bryophytes in order to make them available in bulk amount for their potential use and further bioprospecting [11]. Recently, Decker and Reski [12] have established moss bioreactors for improved biopharmaceuticals The present study aims to establish for the first time stable axenic culture in *in vitro* condition for the moss *Hyophilla nymaniana* and to perform phyotochemical screening. Such knowledge is essential for evaluating therapeutic potential of this moss species.

Material and methods

Plant material and collection site

Healthy, mature sporophytes of *H. nymaniana* were collected from the Birla Institute of Technology campus, Mesra, Ranchi, India. The collected plant materials were identified by Botanical Survey of India Kolkata and National Botanical Research of India, Lucknow and voucher specimens was deposited as the plant herbarium in the department of Bioengineering, BIT Mesra, Ranchi.

Surface disinfection and inoculation of explant

Immature and undehisence capsules of *H. nymaniana* were selected and surface disinfected as follows. Capsules were first washed with distilled water five times to clean off superficial dust, one minute per time. Then the capsules were disinfected with different concentration of NaOCl solution (0.5%, 1%, 2% and 4%) for various time intervals (30s -8min). In order to optimize the concentration time parameter NaOCl treated capsules were

washed with sterile distilled water five times to eliminate remaining NaOCl solution one minute each tern. Spores used for inoculation were released from surface disinfected mature capsules.

Culture medium and culture conditions

The culture medium used in experiment for spore germination consisted of MS medium [13] and basal Knop's medium [14] with varying sucrose concentration (1g/dl, 1.5g/dl and 3g/dl). Spore germination rates were evaluated on a variety of conditions, including nutritive media, light intensity and temperature range. In order to observe the influence of sucrose and mineral salts on spore germination of this species, different strength of MS media and BKP media (Full, 1/2, 1/4th, 1/8th and 3/2 strength) with varying sucrose concentration (0%, 0.5%, 1.5%, 2.0%), were tested. Spores of *H. nymaniana* were inoculated aseptically with the help of sterile needle under laminar air flow cabinet into the above mentioned combinations of media. After the inoculation, three replicates of each medium were maintained under controlled and aseptic condition. Temperature was maintained at 22±1 ˚C. Cultures were provided continuous light of 2000-4000 lux as well as alternate light and dark period of 16 hours and 8 hours respectively with the help of a combination of fluorescent tubes. Once the spores have germinated development of primary protonema observed.

Phyotochemical screening

Chemical tests were carried out on the aqueous extracts of the air-dried powder of moss, using standard procedures, to identify the phytochemical constituents. Crude extracts with different solvents like ethyl acetate , methanol, ethanol of *H. nymaniana* was prepared and chemical test were carried out for the screening of various phytochemicals like flavonoids, alkanoids, glycosides, steroids, terpenoids, tannin, saponin by using protocols of [15,16]with a view to assess their therapeutic values in ethnomedicine.

Test for alkaloids

To a 2 ml of plant extract, few drops of Wagner's reagent were added by the side of the test tube. A reddish – brown precipitate confirmed the test as positive.

Test for flavonoids (Alkaline reagent test)

2 ml of the plant extract was mixed with 2ml of 2% solution of NaOH. An intense yellow colour was formed which turned colourless on addition of few drops of diluted acid which indicated the presence of flavonoids.

Test for phenols and tannins

To 2 ml of the plant extract was mixed with 1ml of 2% solution of $FeCl_3$. A blue-green or black coloration indicated the presence of phenols and tannins.

Test for glycosides

2 ml of the plant extract was mixed with 2ml of chloroform. Then 2ml of concentrated H_2SO_4 was added carefully and shaken gently. A reddish brown colour indicated the presence of steroidal ring, i.e., glycone portion of the glycoside.

Test for saponin

(Foam test)
2 ml of the plant extract was mixed with 5ml of distilled water in a test tube and it was shaken vigorously. No stable foam formation took place indicated for the absence of saponin.

Test for terpenoids

2 ml of the plant extract was dissolve in 2ml of chloroform and evaporated to dryness. To this, 2ml of concentrated H_2SO_4 was added and heated for about 2 minutes. A greyish colour indicated the presence of terpenoids.

Stastical analysis

Stastical analyses were performed to find out the variance among the results and Standard error / Standard deviation were calculated and placed on the graph. The calculations were performed in Excel in Microsoft word 2007.

Results and discussion

This is the first report regarding successful establishment of *in vitro* culture of the moss *H. nymaniana*. The best outcome with reference to surface disinfection resulted from application of 2% sodium hypochlorite solution for (60 s) which leads to lesser contamination without damaging the spores. Almost 90% of the spore germination could be achieved at 2% NaOCl concentration as disinfectant. Although in higher sodium hypochlorite concentration (3% and 4 %) sterilizing efficiency was high but percentage of gametophyte shoot survival was low (Figure 1). Therefore, 2% NaOCl was the best concentration for *in vitro* culture. However, for the other species of moss like *Dicranumscoparium* Hedw. surface disinfection of sporophyte was effective at much higher concentration, 7% of NaOCl solution for 5 minutes [17].

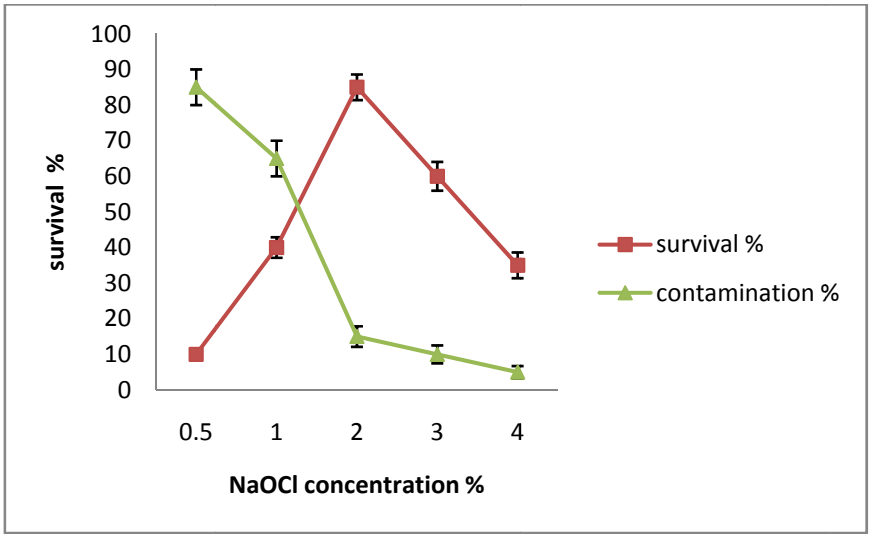

Figure 1: Survival of gametophyte of *H. nymaniana* after treatment with different concentration of NaOCl

Media composition has a key role in modulating the spore germination and protonema development. Maximum spores germination was observed in 1/4th strength MS with 1.5% of sucrose than ½ strength and full strength medium with varying sucrose concentration (Figure 2). Schoefield, 1981[18] reported that most of the bryophyte's spores germinated within 7-30 days after exposure to the favorable condition but in *Plagiochasmaappendiculatum*Lehm. &Lindenb. spores germinated after 10-12 days of inoculation [19]. However, in *H. nymaniana* spore germination occurred bit early (3-4 days) after inoculation.

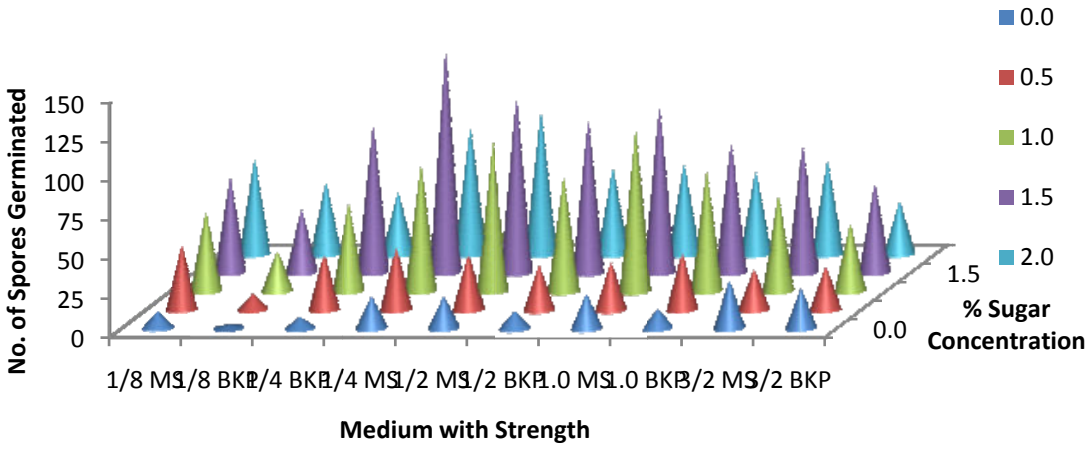

Figure 2: Effect of different combinations of media used for spore germination with varying sucrose concentration.

Temperature and pH also affects significantly the spore germination process. Spores were allowed to germinate at different temperature range from 15-35 C and pH range 4.0-6.5 with continuous light intensity range of 2000-4000 lux at 16/8 hours light condition. Maximum spores germinated at temperature range of 22 ± 1 C (Fig. 3) and at pH 6 in continuous light of 2000-4000lux with alternate 16/8 dark conditions.

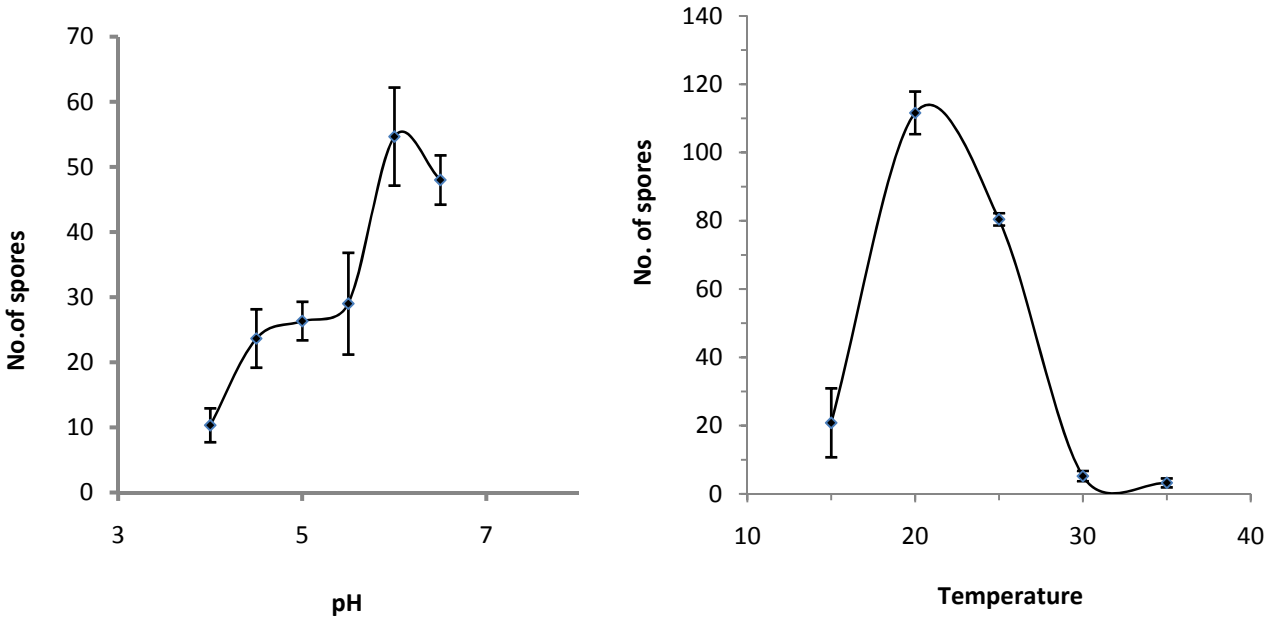

Figure 3: Effect of temperature and pH on spore germination of *H. nymaniana*

Spore germinated within 3-4 days of inoculation (Fig. 4). Primary protonema cells developed rapidly during 10- 15 days of inoculation and chloronemata and caulonemata cells were visible (Fig. 4). Dense protonema appeared like filamentous growth. Regular subculturing was performed to avoid dryness of the medium and the growth of secondary protonema was observed at regular interval. The fully developed gametophyte was observed after 35 days of inoculation.

The results of the qualitative phytochemical screening of the moss *H. nymaniana* showed some positive response as reported in some other bryophyte species. Out of the three extract prepared, the ethanolic extract of the moss showed positive test for alkaloids, terpenoids, steroids and glycosides while the test for tannin and saponin was negative. All the results of the phyotochemical screening of the moss is summarized in Tables 1.Theseresult suggests that the moss plants can be used as potential source of useful drugs in treatment of ailments.

Table 1: Phytochemical screening of *H. nymaniana* in different extract

Test	Ethanolic	Ethyl acetate	Methanolic
Alkaloids	+	-	-
Flavnoids	-	-	-
Tannin	-	-	-
Saponin	-	-	-
Glycosides	+	-	-
Steroids	+	-	-
Terpenoids	+	-	-
Phenols	-	-	-

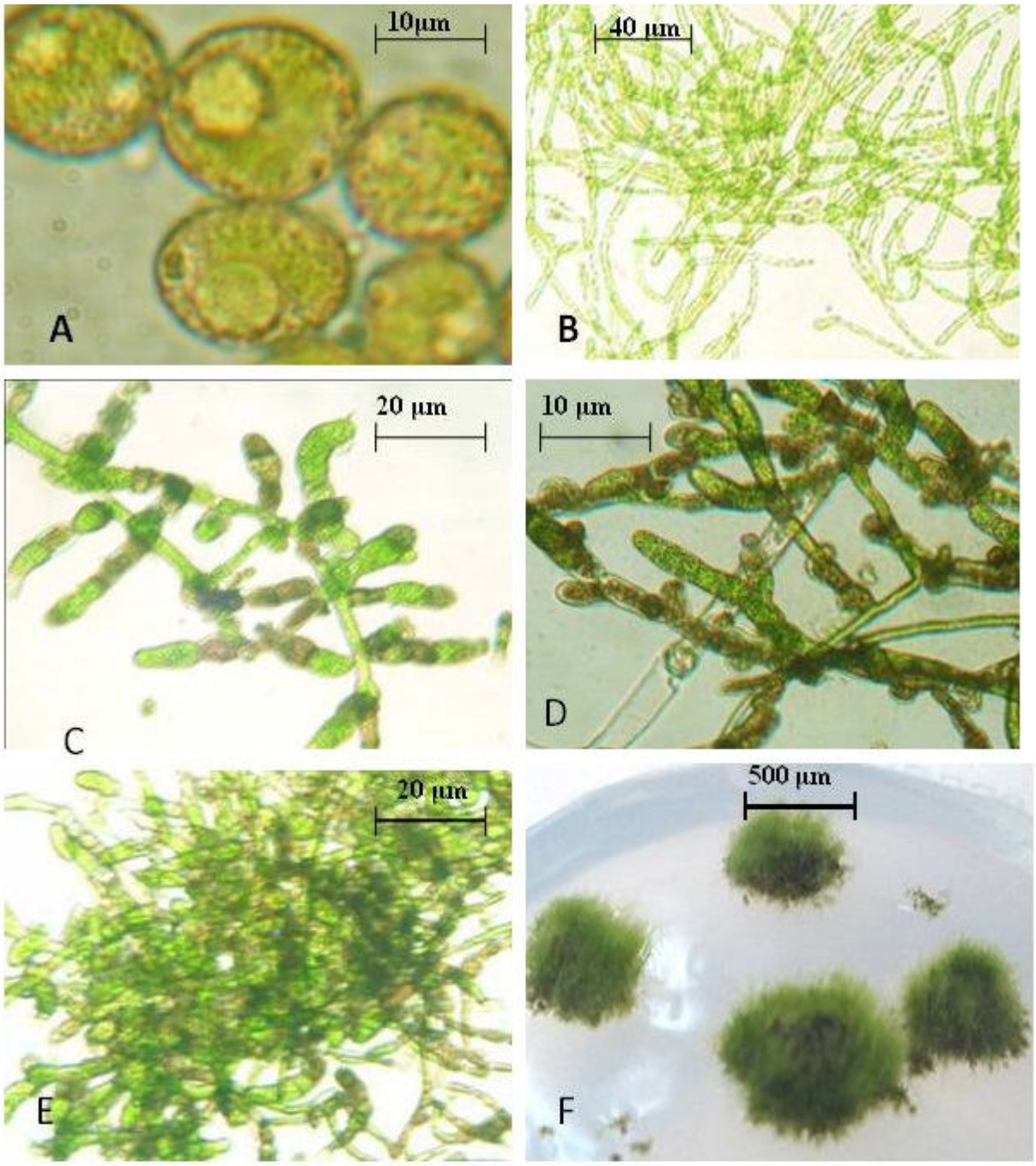

Figure 4: Different stages of *in vitro* culture of *Hyophillanymaniana*(Fleish.)

Menzel (A) Mature spore of *H. nymaniana*. (B) Germinated spore with young protonema 3-4 days old culture. (C) Protonema stage (8 days old culture). (D) Young protonema showing chloronema and caulonema cells. (E) Dense filamentous growth of protonema (25 days of spore germination). (F)Fully developed gametophyte after subculturing on 1/4th strength MS medium with 1.5% of sucrose.

Conclusion

The present study on *in vitro* culture and phyotochemical screening of the moss *H. nymaniana* revealed some biological importance of this species. This is the first successful culture of *H.nymaniana*. Optimization of the culture parameters like culture medium, light intensity, pH and temperature was achieved. Successful protonema culture was established in 1/4th strength MS containing 1.5% of sucrose. Cultured protonema converted into fully developed plant after 35 days of *in vitro* culture. The results of phytochemical screening of cultured moss revealed the presence of some secondary metabolite. But more studies are required to investigate its full therapeutic potential.

Authors contribution

Rashmi Mishra has performed the experiments related to *in vitro* culture study, stastical analysis, phytochemical screening and preparation of manuscript. Vijay Kant Pandey has performed the stasticalanalysis. Dr. Ramesh Chandra has supervised all the work and helped in designing of experiments and editing of the manuscript.

Acknowledgement

Authors acknowledge the support got from UGC MRP Project F No. 37-116/2009 (SR). Authors are grateful for CPDG to RC, Birla institute of Technology, Mesra, Ranchi for providing R&D facilities. Dr. A.K. Asthana NBRI Lucknow and Dr. Md. Nihal Aziz BSI Kolkata are hereby acknowledged for helping in identification of the mosses.

References

[1]. Amarowicz R, Pegg RB, Rahimi-Moghaddam P, Barl B, Weil JA. Free radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chemistry, 2004; 84(4) : 551-562.

[2]. Lee JY, Hwang WI, Lim ST. Antioxidant and anticancer activities of organic extracts from *Platycodon grandiflorum* A. De Candolle roots. Journal of Ethnopharmacology, (2004); 93(2-3):409-415.

[3]. Noccioli C, Meini L, Cecilia Loi M, Potenza D, Pistelli L. A new alpinumisoflavone derivative from *Genista pichisermolliana*. Phytochem. Lett., 2011 ; 4(3) : 342-344.

[4]. Quezel P, Santa S. Nouvelle flore de l'Algérie et des régions désertiques méridionales. Editions

du C.N.R.S, Paris. 1962. Tome I. p. 470-475.

[5]. Giachi I, Manunta A, Morelli I, Pistelli L. Flavonoids and isoflavonoids from *Genista morisii*. Biochemical Systematics and Ecology. 2002; 30(8) : 801–803.

[6]. Tosun F, Kizilay CA, Tosun AU. Flavonoids and isoflavonoids from Genista sessilifolia DC. Growing in Turkey. Chemistry of Natural Compounds. 2009; 45(1): 83-84.

[7]. Pistelli L, Bertoli A, Giachi I, Morelli I, Rubiolo P, Bicchi C. Quinolizidine alkaloids from *Genista ephedroides*. Biochem. Syst. Ecol., 2001; 29: 137–141.

[8]. Erdemoglu N, Ozkan S, Duran A, Tosun F. GC-MS analysis and antimicrobial activity of alkaloid extract from *Genista vuralii*. Pharmaceutical Biology, 2009; 47(1): 81–85.

[9]. Mekkiou R, Touahar H, Dijoux-Franca MG, Mariotte AM, Benayache S, Benayache F. A new isoflavone from *Genista saharae* (Fabaceae). Biochem. Syst. Ecol., 2005; 33 (6): 635-638.

[10]. Boumaza O, Mekkiou R, Seghiri R, Sarri D, Benayache S, Garcia VP, Bermejo J, Benayache F. Flavonoids and isoflavonoids from Genista tricuspidata. *Chem. Nat. Comp.*, 2006; 42 (6): 730-731.

[11]. Boumaza O, Mekkiou R, Seghiri R, Sarri D, Benayache S, Garcia VP, Bermejo J, Benayache F. Benayache. Secondary metabolites from chloroform extract of Genista tricuspidata. *Chem. Nat. Comp.,* 2011; 47 (2): 277-278.

[12]. Mekkiou R, Seghiri R, Boumaza O, Sarri D, chebbah K, Benayache S, Bermejo J, Benayache F. Secondary metabolites from Genista ferox. *Chem. Nat. Comp.*, 2012; 48 (4): 710-711.

[13]. Kerkatou M, Menad A, Sarri D, León F, Brouard I, Bouldjedj R, Chalard P, Ameddah S, Benayache S, Benayache F. Secondary metabolites from *Genista aspalathoides* Lamk ssp. aspalathoides M. Der Pharmacia Lettre. 2013; 5 (5): 285-289.

[14]. Boukaabache R, Boubekri N, Boumaza O, Mekkiou R, Seghiri R, Sarri D, Zama D, Benayache F, Benayache S. Phytochemical study of ethyl acetate extract and antioxidant activity of *Genista quadriflora* Munby (Fabaceae). Der Pharmacia Lettre. 2013 ; 5 (6) : 56-59.

[15]. Rauter AP, Martins A, Borges C, Ferreira J, Justino J, Bronze MR, Coelho AV, Choi YH, Verpoorte R. Liquid chromatography-diode array detection-electrospray ionization mass spectrometry/nuclear magnetic resonance analyses of the anti-hyperglycemic flavonoid extract of *Genista tenera*: Structure elucidation of a flavonoid-*C*-glycoside. Journal of Chromatography A, 2005; 1089: 59–64.

[16]. Rauter AP, Martins A, Lopes R, Ferreira J, Serralheiro LM, Araujo ME, Borges C, Justino J, Silva FV, Goulart M, Thomas-Oates J, Rodrigues JA, Edwards E, Noronha JP, Pinto R, Mota-Filipe H. Bioactivity studies and chemical profile of the antidiabetic plant *Genista tenera*. J. Ethnopharmacol. 2009; 122(2): 384–393.

[17]. Rigano D, Cardile V, Formisano C, Maldini MT, Piacente S, Bevilacqua J, Russo A, Senatore F. *Genista sessilifolia* DC. and *Genista tinctoria* L. inhibit UV light and nitric oxide-induced DNA damage and human melanoma cell growth. Chem. Biol. Interact. 2009; 180 (2): 211–219.

[18]. Harborne JB. Phytochemical Methods. A guide to modern techniques of plant analysis, 3rd Edn. Chapman and Hall, London, 1998; 235.

[19]. Sofowora A. Screening Plants for Bioactive Agents. In: Medicinal plants and Traditional Medicinal in Africa. 2nd Ed. Spectrum Books Ltd, Sunshine House, Ibadan, Nigeria, 1993; pp. 134 - 156. Trease GE, Evans WC. Pharmacognosy. 15th ed. London: Saunders Publishers; 2002.

[20]. Singleton VL, Orthofer R, Lamuela-raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol. 1999; 299: 152-178.

[21]. Quettier DC, Gressier B, Vasseur J, Dine T, Brunet C, Luyckx MC, Cayin JC, Bailleul F, Trotin F. Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. J. Ethnopharmacol. 2000; 72: 35-42.

[22]. Magalhães LM, Santos M, Segundo MA, Reis S, Lima JLFC. Automatic method for determination of total antioxidant capacity using 2;2-diphenyl-1- picrylhyrazyl assay. Anal. Chim. Acta. 2006; 558:310-318.

[23]. Miraliakbari H, Shahidi F. Antioxidant activity of minor components of tree nut oils. *Food Chem.* 2008;111(2):421-7.

[24]. Küçükboyacı N, Özkan S, Tosun F. Gas Chromatographic Determination of quinolizidine alkaloids in *Genista sandrasica* and their antimicrobial activity. Rec. Nat. Prod. 2012; 6:61-64.

[25]. T mová L, T ma J. Dyer's Greenweed (*Genista tinctoria* L.) constituents and biological activity. Ceska Slov. Farm. 2011; Apr 60(2):61-64.

[26]. Rainova L, Nakov N, Bogdanova S, Minkov E, Staneva-Stoytcheva D. Ulceroprotective activity of the flavonoids of Genista rumelica Vel. Phytotherapy Research. 1988; 2(3): 137-139.

[27]. Scarpo R, Paganucci L, Bertoli A, Fiore L, Pistelli L, Federico G. Licoflavone C attenuates the genotoxicity of cancer drugs in human peripheral lymphocytes. Phytotherapy Research. 2008; 22(12): 1650-1654.

[28]. Pietta PG. Flavonoids as antioxidants. J. Nat. Product. 2000 ; 63:1035-1042.

[29]. Rice-Evans. Flavonoids antioxidants. Curr. Med. Chem. 2001; 8: 797-807.

[30]. Luczkiewecz M, Glod D, Baczek T, Bucinski A. LC-DAD-UV and LC-MS for the analysis of isoflavones and flavones from in *vitro* and in *vivo* biomass of *Genista tintoria L*. Chromatographia. 2004; 60: 179–185.

[31]. Mabry TJ, Markham KR, Thomas MB. The systematic identification of flavonoids. Ed. New Yorl, Springer-Verlag, 1970, 354p.

[32]. Aziman N, Abdullah N, Noor Mz, Zulkifli KS, kamarudin WSSW. Phytochemical Constituents and *In Vitro* Bioactivity of Ethanolic Aromatic Herb Extracts. Sains Malaysiana, 2012; 41(11) 1437–1444.

[33]. Queiroz YS, Ishimoto EY, Bastos DHM, Sampaio GR, Torres EAFS. Garlic (Allium sativum L.) and ready-to-eat garlic products: In vitro antioxidant activity. Food Chemistry. 2009; 115(1): 371–374.