European Journal of Biology and Medical Science Research
Vol.4, No.2, pp.69-79, May 2016
Published by European Centre for Research Training and Development UK (www.eajournals.org)

ISOLATION OF ENDOPHYTES AND BIOACTIVE ASSAYS FOR *EMBELIA SPECIES*

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ABSTRACT: *Purpose – The purpose of this study was to compare the bioactivity of three Embelia species in terms of anti-oxidant powers. Emphasis was placed on the high level of anti-oxidant powers towards establishment of cancer therapy. The one with higher anti-oxidant powers was selected for further research. Comparative analysis of endophytes abundance was also done on the assumption that they may be endowing the plants with such anti-oxidant powers. Design/methodology/approach – In this study, laboratory experiments were carried out to determine the phenolic content of the three selected plant species and their comparative antioxidant activities in relation to phenol content through Folin-ciocalteu, DPPH, and ABTS assays. The plant extracts were obtained by sonication and soxhlet method. Thin layer chromatography and HPLC was carried out to help estimate the number of compounds present in the plant species with most activity. Isolation of endophytes from the species on assumption that their presence might be crucial in bestowing the therapeutic value in these plants was also carried out. Findings –The study established that on average, the plant species with higher levels of phenol content had higher activity. E.basal was with high levels of phenol content of 220µg/ml GAE in the sonication extract and 113µg/ml GAE in methanol fraction of soxhlet extraction. At 1mg/ml concentration, DPPH and ABTS assays showed E.basal having highest activity in methanol extract of 61 and 51% respectfully. TLC results showed better separation of bands at hexane: Ethyl acetate ratio of 7:3 respectfully giving eight bands for hexane extract while ethyl acetate fraction gave better band separation at ethyl acetate : hexane : methanol ratio of 3:6:1 respectfully, giving six bands. HPLC gave related results. Different endophyte types were isolated and the most common found was Aspergillus terreus, A.versicolor and Fusarium solani. Research limitations/implications-- The results obtained confirm the therapeutic potency of Embelia. The results suggest that the plant extract possesses certain constituents with antioxidant properties that can be used for the diseases such as cancer. It is assumed that the antioxidant activity shown in this in vitro study by the compounds can have similar antioxidant roles in vivo. This forms a basis for selection of the plant for further investigation in focus towards cancer therapy, the primary long term objective of the researcher.*

KEYWORDS*:* Antioxidants, endophytes, Therapeutic Potency, Radical,

INTRODUCTION

Medicinal plants have become important for the treatment of different disease conditions, such as cancer,diabetes, malaria, among others. They contain bioactive compounds with a therapeutic value. *Embelia* species is one of the medicinal plants that have been adversely used for therapeutic purposes especially among the Indians in Ayuverda. It's on this ground that a need for comparative bioactivity investigation of the plant's three major species arose.

ISSN 2053-406X(Print) ISSN 2053-4078(Online)

Out of the normal cellular metabolic processes and other environmental stresses, free radicals which are unstable molecules, are produced in our bodies and react with cellular components causing cellular and tissue injury, leading to serious diseases such as cancer, inflammation, and diabetes among others. Phenols and Flavonoides are the common plant compounds that have been found to be good antioxidants, compounds that prevent such damage by neutralizing these free radicals. They complement those available in the body like catalase and superoxide dismutase enzymes. Other antioxidants include vitamin C and E.

Solvent extraction is one of the common methods used in the isolation of compounds from plants. Extraction can be either by sonication or soxhlet method among others. They then can be purified through methods like chromatographic techniques including Thin Layer Chromatography(TLC), and High Performance Liquid Chromatography(HPLC).

The objectives to the study were

i) To extract and analyze the antioxidant activity of three *Embelia* species. (*E.ribes, E.tsejericottam* and *E. basal)*

ii) Isolation and characterization of endophytes from the above *Embelia* sp. Fig.1. *Embelia basal* fig.2. *Embelia ribes*

Fig.3.*Embelia tsjeriam-cottam* fig.4 Innoculated plate for endophyte Isolation

REVIEW OF LITERATURE

Embelia is a genus in the Myrsinaceae family and there are about 130 species of the plant.

Embelia tsjeriam-cottam possesses hairy petiolate leaves, while *E. ribes* has ovate smooth leaves and *Embelia basal* a climbing shrub, has generally thick, smooth alternating leaves with entire margins. They are all widely distributed throughout India mainly in Western Ghats. The genus *Embelia* has been investigated for a variety of purposes in Ayurveda (Agarwal, 1997**)**. One of the species, *E. ribes* is used in dental caries. It is also used in the process of formulating anti-AIDS Ayurvedic pharmaceutical compositions. This species shows an antispermatogenic effect and also acts as a contraceptive, (Gayatri, 2011).

Active Constituents:

The initial studies have shown that Embelia contains chemicals embelin, quercitol, and christembin among others. The embelin compound has been reported to induce infertility in mice and dogs by impairing spermatogenesis hence a potential male anti-fertility agent, (Kaul et al, 2002). It has been shown to possess antibacterial activity,((Joshi, 2000) and antiinflamatory activity,((Devaiah,2008). Fresh fruits are eaten as raw for rheumatoid inflammation, (Karuppusamy *et al*, 2009). The study of embelin suggests that, embelin can act as an antioxidant in physiological conditions**,** and with anti-tumor effect,**(**Joy *et al*, 2010).

ISSN 2053-406X(Print) ISSN 2053-4078(Online)

Methods of antioxidant assay

The methods among others, that can be used for the determination of antioxidant potential of plants include FRAP (ferric reducing antioxidant power), DPPH (diphenyl -1, 2- picryl hydrazyl), oxygen radical absorption capacity (ORAC) and 2, 2-azinobis 3-ethylbenzothiazoline- 6-sulfonic acid (ABTS).

Endophytes

Endophytes are microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects. They may benefit host plants by preventing pathogenic organisms from colonizing them. Endophytes can be isolated from host plant by culturing them from a small piece of their host plant in an appropriate growth medium, (Cheplick, 2009). Important aspect of interest is that some plants with bioactive compounds have been discovered to possess associated endophytes that produce the same compounds like in the case with *taxol,* an anticancer agent that is found in yew tree species *Taxus brevifolia* produced by endophytic fungus *Taxomyces andreanae,*(Strobel, 2005).

MATERIALS AND METHODS

Endophyte Isolation and Identification

The current study was carried out to isolate and identify endophytes in selected *Embelia* species.

Media preparation

Water agar media was prepared by accurately weighing 24 g of water agar media bought ready-made and to this 15g of agar was added and homogenised, and autoclaved. Potato Dextrose Agar (PDA) media was prepared by dissolving 24g of PD powder with 15g of agar in 1000ml of sterile water, homogenised, autoclaved as previously mentioned. On cooling to around 450c, antibiotic (streptomycin 100mgl-1) was added and the media aseptically poured into plates. Potato Dextrose Broth (PDB) media was prepared by preparing PD without agar.

Sample preparation and inoculation

The bark samples obtained were surface sterilised by washing under running tap water for 1 hour and then in 70% alcohol for 1 minute. It was then sterilised in 3.5 % (v/v) sodium hypochlorite for 2 minutes and rinsed three times in sterile distilled water for 1 minute. Excess water was blotted out in an airflow chamber using blotting papers. The outer back was removed and the inner portion containing the cortex was carefully dissected into bits of size around 1.0cm x 0.2cm. This were inoculated into water agar medium and incubated in the dark. The stress conditions help to stimulate growth of the endophytes. The plates were monitored regularly for the growth of endophytic fungi.

Endophyte characterization and identification

Endophyte culture plates were selected for identification. Physical morphological characteristics of the various endophytes were noted and portion of the colony scooped onto a microscope slide onto which glycerol drop was added and then a drop of cotton blue stain and

ISSN 2053-406X(Print) ISSN 2053-4078(Online)

the specimen covered with a cover slip and then observed under microscope to study some of its features and in reference to the identification manual, the endophytes were identified.

Bioactivity Assays For E.ribes, E.basal and E.tsjeriamcottam

Collection of plant Samples

The twigs of *E. Basal, E.ribe* and *E. tsjeriam-cottam* were collected. The taxonomic identification was accomplished with the help of a taxonomist. The plants were washed with tap water to remove surface dirt and then dried under shade in open air until attained a crunch/crispy texture.

Preparation of crude extract

Air shade dried stem and leave material were crushed separately to powder in a mixer.

Extraction was done using two methods, i.e sonication method and soxhlet method.

i) Sonication method

10 gm of each plant powdered material was extracted with methanol by the sonication method. The accurately weighed samples were put in well labelled bottles to which 50ml of methanol was added completely immersing the plant extract. This was kept in a shaker for hour at 3000rpm. The samples were then ultrasonicated in an ultrasonicator for 20 minutes, filtered using whatman filter paper and the filtrate left in open air for 24 hours at room temperature to allow solvent evaporate to dryness to get the residual or crude extract. The crude extracts were scrapped into well labelled corresponding centrifuge tubes. These extracts were further used for experimental analysis.

ii) Soxhlet Extraction

In this method, 30g of the sample material was placed in a 'thimble' made of blotting paper in a central compartment of the soxhlet apparatus. The solvents in turn in the increasing order of polarity from hexane, ethyl acetate, methanol and water were used and at temperature between 30-500c . Each solvent was allowed to reflux until the liquefied vapour collecte turned colourless. This took between 8-24 hours depending on the solvent. The apparatus was then disassembled and the extract collected into petri-plates and allowed time in open air for the solvent to evaporate to obtain extract. For each time before change of a solvent, the apparatus was washed well to avoid contamination in extracted fractions. The dried solvent extracts were used for assays.

Total phenolic content estimation

The total phenolic content of the different extracts of plant was determined using the Folinciocalteau reagent method, taking gallic acid as standard according to the method developed by Malik and Singh (Malik, 1980).

The method is based on reduction of the purple radical cation to a blue complex under alkaline conditions. A blue coloured complex is developed due to phosphomolybdic acid, which is present in Folin-Ciocalteu reagent.

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Procedure

The sonication extract samples were diluted on two concentrations i.e 5mg/ml and lmg/ml. 50µl of each extract was mixed with 2.5ml of Fc reagent (1:10 dilution) and incubated for 3minutes and then 2ml of 7.5% Na2Co3 (w/v) was added and mixed well.

The blend was incubated at 45oc for 15 minutes. The absorbance of all the samples was measured at 765nm with 2ml 7.5% sodium carbonate solution in 2.55ml of distilled water as blank.

A standard graph was plotted by taking different concentrations of gallic acid on the x-axis and the absorbance on the y-axis. The concentration of phenol in the given sample was calculated from standard graph of gallic acid. The total phenol content was expressed as gallic acid equivalent (GAE) µg per ml of extract. Experiments were performed in triplicates and results were recorded as mean %.

Antioxidant assays

i) DPPH Assay i.e In Vitro DPPH Scavenging Activity

This antiradical activity assay is based on the reduction of 1,1-diphenyl-2- picrylhydrazyl (DPPH). Due to the presence of an odd electron it gives a strong absorption maximum at 517 nm. The DPPH radical is stable organic nitrogen- centred free radical with a dark purple colour that when reduced to its non-radical form by antioxidants becomes colourless/lightly yellow.

Procedure

In the assay, 95µl of DPPH was loaded in the microplate wells corresponding to the number of samples to be tested including ascorbic acid as standard. To each of the wells with 95µl DPPH, 5µl of different samples at different concentrations to be tested was loaded, and ascorbic acid to one well as a positive control. Methanol was added to one of the wells as a negative control. Two concentrations i.e 5mg/ml and 1mg/ml of the sonication extract samples were used for each sample tested while four concentration levels i.e. 0.5, 1, 3, and 5mg/ml were used for the soxhlet extraction samples. The first and last wells in the series were kept with 100µl of DPPH as control. The procedure was done under low light intensity due to the sensitivity of DPPH to light. The plate was covered with an aluminium foil and incubated in the dark for 30 minutes and absorbance taken at 517nm. Methanol was used as blank.

The free radical scavenging activity (% antiradical activity) was calculated using the equation: % Antiradical Activity = Control Abs. - Sample Abs. × 100 Control Absorbance

ii) ABTS Assay;

Is a decolorization assay applicable to both lipophilic and hydrophilic antioxidants.The preformed radical monocation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), green chromophore that has absorption maxima at wavelengths 734 nm, is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of antioxidants to it colourless neutral form.

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Procedure

The ABTS radical cations are produced by reacting 7mM ABTS and 2.45mM potassium persulphate on incubating the mixture at room temperature in the dark for 12-16 hours. In this, 19.2mg ABTS and 3.3mg potassium persulphate were dissolved in 5ml of distilled water, and kept in dark at room temperature for at least 12 hours to make stock solution. Th solution thus obtained was further diluted with methanol to give an absorbance of 0.70. The spectrophotometer was auto zeroed using methanol as blank. This procedure is according to the method applied by Roberta (Roberta et al 1999), though with some slight modifications. Different concentrations (0.5, 1, 3, and 5mg) of the test samples of 50µl were added to 950µl of ABTS in eppendoff covered with aluminium foil, to give a final volume of 1ml.This was incubated for five minutes. Absorbance was taken at 734nm and percentage radical scavenging activity was calculated as; % inhibition= Control Abs. - Sample Abs. × 100 Control Absorbance

Thin layer chromatography

It gives an estimate of the number of compounds that may be present in the test sample.

Procdure

The TLC plates were prepared by weighing 30g of silica and dissolving it in 65ml of water. The paste so formed was thinly and uniformly spread on the glass slides and allowed to air dry before put in a hot air oven for activation overnight at 1000C.

The activated silica gel slides were used to run chromatograms of the various plant samples. In this, a spot was made at 1cm from the bottom edge of the slide and allowed to dry. Meanwhile, solvent systems of different ratios were prepared in the tissue culture bottle lined from the inside with a blotting paper and allowed to equilibrate. The spotted pencillabelled silica slide were then put in the solvent systems and allowed time for the run. When the solvent reaches close to the opposite edge of loading, the slide is carefully taken out an observed for the bands. The slides were further developed using iodine vapours. The bes solvent-combination ratio was determined depending on the number and clarity of the bands produced. Several solvent systems were assessed for their capacity to resolve the components of the extracts.

Fig.6 *Solvent trials in thin layer chromatography for best band separation*

HPLC

The soxhlet extracts of methanol, ethyl acetate and water were subjected to HPLC finger print study. Thermo hypersil-C18 column was used. The mobile phase components were 0.1%H3PO4 in water and acetonitrile in the ratio 65:35 respectfully, pumped from the solvent reservoir at an injection volume of 20uL.

RESULTS

Endophytes

The endophytes emerging were unique in morphology .Endophytic fungal strains were identified based on morphological characters using standard identification manual. *Aspergillus*

ISSN 2053-406X(Print) ISSN 2053-4078(Online)

terreus, *A.versiclor*, *Bipolaris spicifera*, *Pestalogia sp* and *Fusarium solani* were the dominant fungal endophytes identified. It was aimed that the secondary metabolites of the endophytes was to be processed and assessed to check if there is any activity in relation to the plant extracts to help establish the assumption that the therapeutic properties of plants might be due to the secretion of secondary metabolites by the endophytes residing in this plants.

Due to time constraint at the moment, this was not achieved but it remains a priority of investigation in the near future.

Aspergillus versicolor Aspergillus sp. Pestalogia sp

Fig.7: Endophytes isolated, characterised and identified

Total phenolic content estimation

Total phenol content varied in the different plants and in the different solvents. In the sonication extract, at 1mg/ml concentration, *E. basal* had the highest phenol content of GAE of 220µg/ml, while it had 113 µg/ml in soxhlet extract, and the content of phenol in the plant extracts appeared to be directly proportional to extract concentration. Methanol extract in soxhlet, showed highest content of phenols in all concentrations.

Fig.8 Total phenol estimation chart

Determination of antioxidant activity

Measurement of DPPH radical scavenging activity

The antioxidant abilities of the various Embelia sp.samples was determined as the ability of the extract to scavange DPPH radicals according to the method of Bondet (Bonde et al,1977). The decrease in absorbance due to scavenging for DPPH radical by the plant extracts was determined at 517nm using UV-visible spectrophotometer after 30minute incubation. There was high level of decolouration of the DPPH with increased phenols. Th reduction in DPPH radical is a measure of antioxidant activity. A significant decrease in concentration of DPPH radical was found with increase in concentration of plant phenoli content.

Fig.11 DPPH assay plate showing different levels of decolourization

Fig.9. DPPH sonication extract

Measurement of ABTS radical scavenging activity

ABTS is designed to measure the overall antioxidant capacity within a given sample

to inhibit the oxidation of ABTS in comparison to Ascorbic acid.

Fig. 11 ABTS assay chart

Fig.12 Standard ascorbic acid curve- ABTS

4.4 Thin layer chromatography

Several different solvent ratios were tried to establish the fit combination of solvent that can achieve a high level of clarity in separation of the phenolic compounds present in the sample. A combination of ethyl acetate to hexane in the ratio of 7:3 respectively showed the best

ISSN 2053-406X(Print) ISSN 2053-4078(Online)

separation in ethyl acetate extract sample giving six bands. On development in iodine vapours, clarity of some bands improved but no any noticeable increase in the number of bands.

»»»»»»»»» *set A*

»»»»» » » *set B*

Fig.13 Ethyl acetate extract in ratio of 3:6:1 of ethyl acetate:hexane: methanol gave better band separation as compared to other ratios.

Standardisation of methanol extract did not work out well despite a 6-solvent system range tried individually and in combination. No clear band separation was achieved in methanol extract.

HPLC

HPLC profile for ethyl acetate extract showed the compounds separated into two major peaks with many other small minor peaks indicating the presence of two major compounds. The major compounds separated at retention time of 13.185min and 15.870minutes.

Fig.14 Ethyl acetate fraction HPLC profile.

Methanol fraction HPLC profile gave two major peaks. The profile showed compounds separated at a retention time of 13.359min and 15.930minutes. The peaks were selected at a wavelength of 280nm due to sharpness of the peaks and proper baseline. The profile is almost identical to ethyl acetate fraction.

Fig.15 . Methanol fraction HPLC profile of E.basal.

Water fraction profile gave three prominent peaks selected at a wavelength 280nm due to sharpness of the peaks and proper baselines. The compounds separated at retention time 13.491min, 14.660min and 15.870min.

Fig16 HPLC fingerprint profile of aqueos extract of E.basal

DISCUSSION AND CONCLUSION

Discussion

Embelia basal is a well known traditional medicinal plant. Its berries, root, barks and leavesare useful for medicinal purposes. Embelia species are used in Ayurvedic system of medicines and mainly promoted as antibacterial as well as anthelmintic mediator. On comparing the total phenolic content and DPPH assays on all the three initially chosen Embelia species for study, Embelia basal showed better results. Therefore this was selected for further investigation to determine its therapeutic potential in terms of antioxidant ability through soxhlet extraction and identify if there are differences in activity depending on extraction method.

Extracts were analysed for their antioxidant activity and total phenolic content establishing any relationship if any between them and in which case it was realised that methanol extract showed promising activities. Activity was also established to positively correlate with the total phenolic content of the extracts.

ISSN 2053-406X(Print) ISSN 2053-4078(Online)

In DPPH radical scavenging ability of extracts, Embelia basal was found to have a high activity among the three species when compared at 1mg/ml concentration of sonication extract. This was at 58% inhibition. In the soxhlet extract, both the solvent samples of hexane, ethyl acetate, methanol and water were found to posses potential to scavenge DPPH and ABTS radicals, with an assumption then, that the active compound present will be both polar and non-polar.

TLC results showed that there are more than ten compounds more of which are polar that act synergistically giving the antioxidant property.

The HPLC profiles showed the presence of basically three major compounds in the plant though it has several minor compounds that didn't produce prominent peaks. The HPLC results confirms the TLC results that showed the presence of many compounds in terms of bands produced. Further advanced spectroscopic studies are required for structural elucidation and identification of the compounds detected in the plant as well as to establish which of the compounds has the activity.

This results compared to an early work that has been done to some extent correlate. Ganesan(2009),recorded ethanol extract of Embelia as having antioxidant and anticancer property.

A variation arises however with the work recorded by Mhaskar et al (2011),that among the three species, E. basal has the lowest embelin content of 1.6% as compared to E.ribes with 2.3 to 3.3%, with the inference from this observation that E.ribes has high phenol content and activity than E. basal. It however remains an area of further study to establish if the locality/ region from where a plant is obtained can be a cause of variation, a point that the current study assumes to cater for the disparities realised in the present study with the early work.

Sample Total phenol
µg/ml GAE
DPPH%
Activity
ABTS%
activity
TLC Best
solvent ratio
TLC
bands
ERL 125 14
ERB 150 43
ETL 40 17
ETB 160 44
EBL 223 58 Chosen for further analysis as below
EBB 130 17
Analysis Of Embelia basal soxhlet extract
HX 81 43 44 7HX:3EA 8 bands
EA 78 51 51 3EA:6HX:1MEOH 6 bands
MEOH 113 61 53 Standardization failed
WATER 70 36 36 Not tried
Fig.18 Summary table; all compared at 1mg/ml concentration

ISSN 2053-406X(Print) ISSN 2053-4078(Online)

CONCLUSION

This result indicates that the direct scavenging of free radicals cannot be ruled out in the mechanism of therapeutic function of this plant species. In addition, the *Embelia basal* species has been evaluated for antibacterial activity and found positive for several bacterial strains and fungi, (Gayatri S. et al (2011)).

Though Embelia has free radical scavenging activity, it has to be evaluated for other pharmacological properties. The results obtained confirm the therapeutic potency of Embelia used in traditional medicine. In addition, these results form a good basis for selection of the plant for further phytochemical and pharmacological investigation. The present study supports and indicatively suggests that the plant extract possesses certain constituents with antioxidant properties that can be used for the diseases such as cancer.

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