EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 4
Issue: 6
214-220
Year: 2017

PHARMACOGNOSTIC AND ANTIMICROBIAL SCREENING STUDIES OF *EUGENIA JAMBOLANA* SEEDS

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Article Received on 21/03/2017 **Article Revised on 11/04/2017** **Article Accepted on 01/05/2017**

ABSTRACT
Eugenia jambolana is a large evergreen tree indigenous to the Indian subcontinent belonging to the family Myrtaceae. Plants are rich in a variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, phenols, steroids, glycosides and volatile oils that serve as natural plant defence mechanisms against invasion by microorganisms, insects, and herbivores or for combating infectious or parasitic agents or generated in response to stress conditions (Cowan, 1999). *Eugenia jambolana* have promising therapeutic value with its various phytoconstituents such as tannins, alkaloids, steroids, flavanoids, terpenoids, fatty acids, phenols, minerals, carbohydrate and vitamins. In a study, the antimicrobial activity of *E. jambolana* leaves extract was done against clinical isolates by using well diffusion method. The different extracts showed inhibitory activity against clinical isolates of gram negative bacteria such as *Salmonella typhi* , *Shigella dysentriae*, *Kelbisiella pneumonia*, *Pseudomonas aeruginosa*, *Ecoli*, and gram positive bacteria such as *Bacillus subtilis*, *Staphylococcus aureus* (Jetthu *et al.*, 2011; Parkash *et al.*, 2011). Antimicrobial activity using (Agar well diffusion assay). Amongst various extracts (EJPE, EJC, EJM and EJAQ) of *E. jambolana* tested, methanol extract (EJM) exhibit significant antimicrobial activity. Finally, bioactive extract was subjected to the chromatographic separation of chemical compound which was further characterized by using spectroscopic method and the isolated compound was found to be 3-(2,2,3,3,4,5,5,6,6-nonamethylcyclohexyloxy)-3,4-dihydro-2.

KEYWORDS: *Eugenia jambolana* seeds, Pharmacognostic, Antimicrobial study, Medicinal plants.

1. INTRODUCTION
The great Northern region of India and its herbal richness cater to the needs of modern medicine. This diversity of the Northern region, if used effectively and scientifically, it can provide a new drug molecule which may combat the adverse effects of the synthetic drugs used worldwide as well as reduce the cost of the medication. So, these facts if used further can be useful in treating many dangerous diseases. Especially, herbal plants have proven themselves a strong contender in acting as natural resource for bioactive compounds, such as *Taxus brevifolia* (Houghton *et al.*, 2007), *Gingko biloba* (Goktas *et al.*, 2007). Therfore, the herbal plants got importance and became essential ingredients of the various medicines of traditional Indian system of medicine such Ayurveda, Unani and Homeopathy. Besides the availability of reports on analgesic activity of some indigenous medicinal plants, the medicinal flora of our country still remains virtually unexplored. Thus, in an attempt to develop potent medicinal agent to fight against infectious diseases and related pain, in the present study, evaluation of analgesic activity of seeds of Jamun (*Eugenia Jambolana* Lam.) is carried out. It belongs to the family Myrtaceae and is native to the Indian subcontinent. Jamun tree is famous for its different names and for its fruit called berries. Annually, this tree produces oblong and ellipsoid fruits called berries. They are green, when raw and purplish black, when fully ripe and are sweetish sour to taste and contains high percentage of nutrients such are minerals, vitamins, tannins and protein content in adequate amount (Paul and Shaha, 2004). The pharmacologically active phytoconstituents present are flavanoids, terpenoids, anthocyanins and tannins (Li, Zhang and Seeeram, 2009). Jamun is a plant with known ethnomedicinal uses. Before the discovery of Insulin, jamun was highly useful in the treatment of diabetes and is an integral part in various system of medicine (Helmstadter, 2007). The plant has gained importance as an herbal drug to cure several diseases such as viral infections, inflammatory disorders, allergic disorders, gastric ulceration, heart diseases, cancer, liver infections, diarrhoea and diabetes. But the analgesic activity of *Eugenia jambolana* are not yet explored. So, it was decided to study these activity

with the seeds of *Eugenia jambolana* using various animal and experimental models.

2. MATERIALS AND METHODS

2.1 Plant Material

The seeds of *E. jambolana*, commonly known as Jamun, were collected from the botanical garden of Guru Nanak Dev University (GNDU) campus in the month of July 2011 and were then dried (Figure 5). The taxonomic identification of the plant was confirmed by Mr. Ram Prasad, Department of Botanical & Environmental Sciences, GNDU, Amritsar. A voucher specimen no. (No. 29) herb has been deposited in department's herbarium.

2.2 Chemicals and Solvents

2.2.1 Solvents

Petroleum ether (60-80ºC) (Qualigens Fine Chemicals, Mumbai); Chloroform (Qualigens Fine Chemicals, Mumbai), Methanol and Ethanol (Qualigens Fine Chemicals, Mumbai) were used for the extraction of required plant material and the subsequent analysis.

2.2.2 Chemicals

Chloroform, Sulphuric acid, hydrochloric acid, Mayer's reagent, Dragendorff's reagent, magnesium turnings, hydrochloric acid, ammonia solution, benzene, pyridine, sodium nitroprusside, sodium hydroxide, ferric chloride were used for the phytochemical screening of the plant extracts.

2.3 Pharmacognostic Standardization

2.3.1 Organoleptic Features

The plant was observed with the naked eye for its varied features like color, odour, taste, size, shape and other features like touch and texture of the seeds of *E. jambolana*.

2.3.2 Microscopic Examination

2.3.2.1 Powder study

The dried seeds were powdered and was cleared in chloral hydrate solution, stained with weak iodine solution, phloroglucinol-HCl, then mounted with dilute glycerine and observed under a light microscope (Ruzin, 1999).

2.3.2.2 Photomicrograph

The microscopic descriptions of the selected tissues were supplemented with micrographs. The photomicrographs of the different magnifications were taken with Olympus Magnus Microscope and for the normal observations, a bright field was used.

2.4 Ash Values

The material remaining after incineration of the powdered drug material is called as ash content of the drug, indicating the presence of the inorganic salts naturally occurring in the plant drug or due to the adulteration of available plant material. The ash values were determined by using total ash, water soluble ash and acid insoluble ash as per the procedure given in Indian Pharmacopeia (IP, 1996) and WHO guidelines (WHO, 1998).

2.4.1 *Total Ash*

This method is used to measure the total amount of the material remaining after ignition (silicates, carbonates, etc). This material obtained may include 'physiological ash' which is derived from the plant tissue itself plus 'non-physiologiacl ash' which is the residue of the powder extraneous matter as sand, soil, etc. adhering to the surface of the plant. Absolutely air-dried powdered drug (approx. 2-4 g) was weighed in a silicon crucible and was incinerated at a temperature not exceeding 400ºC in a muffle furnace (Narang scientific works, New Delhi) until it is devoid of carbon or when it appears white in colour, cooled and then weighed. The percentage of ash with reference to the air-dried drug was calculated (WHO guidelines, 1998).

2.4.2 *Acid Insoluble Ash*

The acid insoluble ash is the residue left after boiling the total ash content obtained above with 25 ml of 2 M hydrochloric acid and subsequently igniting the insoluble matter for 5 minutes. The resultant matter was collected in silicon crucible or most probably on an ash-less filter paper washed with hot water, ignited, cooled in a dessicator and accurately weighed. The percentage of acid insoluble ash with reference to air-dried drug was calculated (WHO guidelines, 1998).

2.4.*3 Water Soluble Ash*

The total ash content was boiled in a china dish containing 25 ml of distilled water for 5 minutes. The resultant insoluble matter left was collected in a sintered crucible or on an ash-less filter paper, washed with hot water and ignited in a crucible (Galaxo, Mumbai) for 15 minutes not exceeding 400ºC. Finally, calculate the difference between the weight of the residue (in mg) from the weight of total ash. The percentage of water-soluble ash with reference to air-dried drug was calculated (IP, 1996).

2.5 Loss on Drying (LOD)

Oven-dried glass-stoppered weighing bottle was weighed. Subsequently, the bottle and the powdered sample material were accurately weighed. The stopper was removed and the bottle was placed in an oven until the sample was dried to a constant weight. When absolutely dried, immediately the bottle was stoppered and was allowed to cool to room temperature while kept in a dessicator. Finally, the bottle and its contents were weighed. Loss on drying was evaluated by calculating the differences of the two (before and after drying) weighings (WHO guidelines, 1998).

2.6 Determination of Extractive Value

2.6.1 *Alcohol Soluble Extractive Value*

Alcohol is an ideal solvent for extraction of various phyto-chemicals as tannins, resins, etc. thus, this method

is used to determine the approximate resin content of drug. The powdered drug material (approx.4-5 g) was macerated with 25 ml of ethanol in a closed conical flask for 24 hours. For first 6 hours. Shake the contents of the flask frequently and for the next 16 hours, the flask was allowed to stand still, without any disturbance. The resultant extract was filtered. The filtrate (25 ml) obtained was evaporated to dryness and the residue obtained was dried at 105ºC and finally weighed. The percentage of ethanol-soluble extractive with reference to the air-dried drug was calculated (Ayurvedic pharmacopoeia of India, 2008).

2.6.2 *Water-Soluble Extractive Value*

This method can be applied to plant drugs containing water soluble constituents of crude drugs as tannins, glycosides, sugars, plant acids and mucilage, etc. 4-5 g of the powdered drug material was added to 25 ml of water (80ºC) in a tightly closed conical flask and kept in mechanical shaker. After vigorous shaking, it was allowed to stand still for 18 hours. The filtrate obtained (25 ml) was evaporated to dryness and the residue obtained was dried at 105ºC and finally weighed. The percentage of water-soluble extractive with reference to the air-dried drug was calculated (WHO guidelines, 1998). Similarly, the petroleum ether, chloroform and ethyl acetate extractive values of the powdered plant material were carried out.

2.7 Preparation of Extracts

The seeds of *E. jambolana* were dried in shade and coarsely powdered. Approx. 1.5 Kg of the powdered material was subjected to successive Soxhlet extraction using different solvents in an increasing order of their polarity viz. petroleum ether (60–80ºC), chloroform, methanol and distilled water for not less than 48 hours. After each extraction, the powdered material was dried in air at room temperature. Finally, marc was digested with distilled water for 24 hours or more to obtain the aqueous extract (Figure 6). Each extract was concentrated in-vacuo using rotatory evaporator. Extracts were weighed subsequently and the percentage yields were calculated of each extract obtained individually in terms of the air-dried weight of the plant material.

2.8 Preliminary Phytochemical Screening

The extracts of *E. jambolana* were tested for the presence of various phytochemicals such as alkaloids, glycosides, tannins, steroids, saponins, flavonoids, etc. (Kokate, 2008; Khandelwal, 2006).

2.9 Fluorescence Analysis

The powdered seed material was analyzed under visible light, short ultraviolet light, long ultraviolet light after treatment with various organic / inorganic reagents like sodium hydroxide, nitric acid, ammonia, etc (Pratt and Chase, 1949).

3. EVALUATION OF ANTIMICROBIAL ACTIVITY

The present study has been carried out to assess the antimicrobial activity of crude extracts of dried seeds of *E. jambolana* against four selected bacterial strains and two selected yeast strains. The antimicrobial activity of crude extracts has been evaluated on the basis of Agar well diffusion assay (Bauer *et al.*, 1966).

3.1 MICROORGANISMS (BACTERIAL AND YEAST STRAINS)

The reference strains of the clinically important bacteria and yeast were obtained from Institute of Microbial Technology (IMTECH), Chandigarh through Department of Microbiology, Guru Nanak Dev University (GNDU), Amritsar.

1	**Bacteria**	**Reference number**
1.	*Staphylococcus aureus*	MTCC-740
2.	*Klebsiella pneumonia*	MTCC-109
3.	*Salmonella typhimurium*	MTCC-98
4.	*Methicillin resistant Staphylococcus aureus*	MTCC-108

Yeast strains

1.	*Candida albicans*	MTCC-3017
2.	*Candida tropicalis*	MTCC-230

All the reference strains were maintained on nutrient agar slants, except the two yeast strains (*C. albicans* and *C. tropicalis*) which were maintained on yeast malt agar (YMA) and sabaurand agar, respectively; subcultered regularly and stored at 4^{o}C as well as at -80^{o}C.

3.2 MAINTAINENCE OF CULTURE

The cultures were maintained on nutrient agar slants. All cultures were stored at 4°C. The stock cultures were subcultured at regular intervals.

3.3 PREPARATION OF VARIOUS MEDIUMS FOR BACTERIAL GROWTH

The following mediums were prepared and stored appropriately:

Table1: Muller Hinton Agar Media (HI MEDIA Laboratories, Mumbai)

S. No.	Ingredient	Gm/L
1	Casein acid hydrolysate	17.5 g
2	Beef heart infusion	2 g
3	Starch soluble	1.5 gm
4	Agar	17.0 gm
5	Distilled water	1000 ml
6	pH	7.4 ± 0.2

Thirty eight grams of commercially available Muller Hinton agar was suspended in 1000 ml of distilled water and boiled to dissolve the media completely before autoclave.

Table 2: Nutrient Broth Media

S. No.	Ingredient	Gm/L
1	Peptone	5 g
2	Sodium chloride	5 g
3	Beef extract	1.5 g
4	Yeast extract	1.5 g
5	Distilled water	1000 ml
6	pH	7.0 ± 0.2

Table 3: Sabourand Agar

S. No.	Ingredient	Gm/L
1	Peptone	10 g
2	Dextrose	40 g
3	Agar	15 g
4	Distilled water	1000 ml

Table 4: Yeast Malt Agar

S. No.	Ingredient	Gm/L
1	Peptone	5 g
2	Yeast extract	3 g
3	Malt extract	3 g
4	Dextrose	10 g
5	Agar	15 g
6	Distilled water	1000 ml

3.4 STERILIZATION

All the media, distilled water and glassware were sterilized in an autoclave (Calton, vertical autoclave, Narang Scientific works, India) at 15lb pressure for 20 minutes.

3.5 PREPARATION OF INOCULUMS

A loopful of isolated colonies of different bacterial and yeast strains were inoculated into 5 ml of nutrient broth, Yeast malt broth and Saboraund broth and incubated at 37ºC and 25 ºC, respectively in an incubator shaker (New Brulsiick Scientific, Edison NJ, USA) for 4 h at 300 rpm. The inoculum, thus prepared was used further for antimicrobial sensitivity testing.

3.6 PREPARATION OF EXTRACT SAMPLES FROM DRIED RESIDUES

DMSO is very good solvent for experimental purpose. So it was used for dissolving various extracts obtained as a result of soxhlet extraction for antimicrobial testing. Variable concentrations of extracts were prepared by dissolving dried residues in DMSO for testing inhibitory efficacy against selected bacterial strains. Stock solutions of extracts were diluted in DMSO to produce concentrations ranging from 50 to 200 µg/ml.

3.7 SENSITIVITY OF BACTERIA TO DIFFERENT PLANT EXTRACTS BY AGAR DIFFUSION METHOD

The sensitivity of different bacteria and yeast to plant extract was measured in terms of zone of inhibition using agar well diffusion assay (Bauer *et al*., 1966). The plates containing Mueller-Hinton, Sabouraud and Yeast malt agar were uniformly spread with 0.1 ml of the inoculums of different bacteria and yeast. Wells (8mm diameter) were cut out from agar plates using a sterilized stainless steel borer and filled with 100µl of the extract (aqueous/organic). The plates inoculated with different bacterial and yeast strains were incubated at 37ºC and 25 ºC upto 24 h and the diameter of any resultant zone of inhibition was measured. The experiment was performed in duplicate. Further the antimicrobial activity was compared with two standard antibiotics - Ampicillin (10 mcg) and Chlorampenicol (30 mcg).

3.8 EFFECT OF DIFFERENT CONCENTRATIONS OF EJPE AND EJM EXTRACTS OF *E. JAMBOLANA*

Agar diffusion assay was carried out at different concentrations (100, 150 and 200 mg/ml) of different extracts petroleum ether, chloroform, methanol and aqueous extracts in a similar manner as described above and DMSO, the vehicle used to dissolve the extracts, was considered as control. The maximum activity was found in methanol extract.

3.15 COLUMN CHROMATOGRAPHY OF BIOACTIVE EXTRACT

The bioactive methanol extract (28 g) of *E. jambolana* seeds was loaded onto the column packed with Silica gel (60-120 mesh; Titan Biotech Ltd.) and was eluted using chloroform until 61 fractions and then the polarity was increased by using chloroform: methanol at different ratios. The obtained solution was distilled on the water bath, until 2-4 ml of the fraction is left, which is then collected in a 5 ml vial. Each fraction was simultaneously spotted on the Silica gel-G prepared TLC (Thin layer chromatogram) plates using chloroform and methanol as mobile phase at the increasing polarity. A total no. of 659 fractions was collected by the similar method.

3.16 CHARACTERIZATION OF FRACTIONS

The fractions with the similar chromatographic pattern obtained on TLC plates were characterized by using:-

1. ^{1}H- NMR (300-MHz Brukel and Jeol NMR spectrometer) and ^{13}C- NMR (75-MHz Brukel and Jeol NMR spectrometer) spectroscopic analysis;
2. Infra-red (IR) spectroscopic analysis (FTIR Thermospectrophotometer);
3. Mass spectroscopic (MS) analysis [HRMS (ESITOF) Mass spectrometer].

4. STATISTICAL ANALYSIS

The data is expressed as mean + S.E. (Standard error mean) and the data was analysed by one way ANOVA followed by student's t-test *$p<0.001$ vs control.

5. DISCUSSION

There are extensive and diverse natural resources that are available for production of useful products such as antibiotics (Fox and Howlell, 2008). The bacterial and yeast cultures used in the present study were responsible for causing gastrointestinal tract infections, respiratory

and skin infections. The worldwide spread of Methicillin-resistant *S. aureus* (MRSA) has been observed (Talbot *et al.*, 2006). Multidrug resistant pathogens, especially bacteria, emerged as a major therapeutic challenge complicating the antibiotic therapy regime. It prompted the discovery and development of new antibiotics to combat difficult-to-treat infections caused by such pathogens (Livermore, 2009). This situation coupled with the undesirable side effects of certain antibiotics and the emergence of previously unknown infections leading to the failure of chemotherapeutics has necessitated a search for new antimicrobial and chemotherapeutic agents that combine antimicrobial efficacy with low toxicity and minimum environmental impact (Song, 2008). The present research was focused on the seeds of *E. jambolana* extracts for the pharmacognostic studies such as fluorescence analysis, ash values, extractive values, loss on drying, etc. as per the Indian Pharmacopoeial guidelines. The total ash was 2.43%, acid insoluble ash was 1.5%, water soluble ash was 1.7% and loss on drying was 15%. The extracts of *E. jambolana* which are best effective in antimicrobial activities are in conjunction with the phytochemical constituents present in the seeds of the plant. Thus, the phytochemical screening of *E. jambolana* extracts revealed that the petroleum ether extract contains terpenoids, chloroform extract contains alkaloids and tannins, methanol extract contains flavonoids, tannins, glycosides, saponins and alkaloids were present in aqueous extract. A tentative anthocyanin compound was isolated from bioactive methanol extract by the column chromatography and confirmed by NMR, IR and Mass spectroscopic analysis. Therefore, these phytochemicals are speculated to account for the observed pharmacological effects of the plant's extract. Many authors have reported that the phenolic compounds such as flavonoids, tannins, cyanins, triterpenoids and other phenolic compounds possess multiple biological activities such as antinociceptive and inhibitory action on arachidonic acid metabolism (Kumar *et al.*, 2008; Muruganandan, 2001).

The seeds of *E. jambolana* were evaluated for antimicrobial activity against four reference strains of bacteria, including two Gram-positive and two Gram-negative bacterial strains and two yeast using agar diffusion assay. The study revealed petroleum ether and methanol to be the best solvent to isolate the compound responsible for antimicrobial activity while aqueous and chloroform extract was resistant to all the test organisms. Our results are in consonance with Mashhadian and Rakshandeh (2005) according to which methanol extract gave better results than aqueous extracts which was ineffective. *Staphylococcus aureus*, one of the major bacterial pathogens of man, causing a variety of diseases and also the most common cause of nosocomial infections (Prakash *et al.*, 2007) showed the better sensitivity to plant extracts in comparison to the standard antibiotics. The extracts at 150 mg/ml concentration showed its best activity. Although chloramphenicol was active against all test organisms but ampicillin was not active against *K. pneumonaie*. Similarly, the methanol and petroleum ether extract of *E. jambolana* effective against *K. pneumonaie* which is responsible for respiratory infections (Annamalai *et al.*, 2011).

^{1}H- NMR shows the signals at aromatic region at range δ 7.61-6.81 with doublets having ortho-coupling (j = 8.1 Hz) and some resonance in proton spectrum showed at δ 5.54-4.25, due to coupling of glycosidic protons. A singlet at δ 3.55 appeared due to methoxy group and singlets of methyl group appear at δ 1.88 and 1.25 ppm, respectively. ^{13}C- NMR of this unknown compound showed various resonances at the region of aromatic δ 100-150 and aliphatic region δ 20-60. It means the unknown compound may have both aliphatic and aromatic groups. This methoxy group, already assigned by ^{1}H spectrum, further revealed a peak at the region δ 58.5 ppm by ^{13}C- NMR. Its IR spectrum shows the presence of OH group because of the stretching of OH which appeared in the IR spectrum at 34.22 cm^{-1}. It may be intermolecular H- bonding and other strong stretching at 1471 cm^{-1}, due to C-O linkage. According to the above analysis, the unknown compound may be anthocyanin. The tentative structure is finally confirmed by its base peak which appeared in the mass spectrum at 371 M^{+}. From the spectroscopic analysis, it was observed 3-(2,2,3,3,4,5,5,6,6-nonamethylcyclohexyloxy)-3,4-dihydro-2-(4methoxyphenyl)-2H-chromene-7,8-diol be the tentative structure of the isolated compound.

The anthocyanins present in *E. Jambolana* seed may be responsible for antimicrobial activity. Cesoniene *et al* (2009) reported that the European Cranberry extracts inhibited the growth of wide range (Gram-positive and gram-negative) of human pathogenic bacteria due to the highest quantity of anthocyanins present in the cranberries. Zhao *et al* (2009) investigated the antimicrobial activity of extracts of purple corn (*Zea mays* L.) and found it due to the high level of anthocyanins in *Zea mays* as confirmed by several assays. Gonzalez and Stasi (2002) reported the analgesic activity of *Wibrandia ebracteata* due to the presence of polyphenolic compounds such as anthocyanins and flavonoids. Miladiyah *et al* (2011) reported that *Manihot esculenta* leaves possess analgesic activity, mainly because of the presence of anthocyanin compounds. Therefore, the anthocyanins are responsible for antimicrobial activity.

6. ACKNOWLEDGEMENT

I would like to thank my supervisor, Dr. Hayat M. Mukhthar for his supervision from **Shaheed Bhagat Singh College of Pharmacy, Patti, Amritsar 143416, Punjab, India,** for providing me the opportunity and all the necessary facilities to complete this endeavour successfully.

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