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PHYTOCHEMICAL INVESTIGATION AND BIOLOGICAL ACTIVITIES OF *GMELINA ARBOREA*

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ABSTRACT

To initiate the work 5 medicinal plants were identified and screened for antimicrobial activities against test organisms and antioxidant potential. Out of these plants Gmelina arbor Traditional knowledge of medicinal plants has always guided the search for new cures. simple medicinal preparations often mediate beneficial responses due to their active chemical constituents ea showed potential antimicrobial as well as antioxidant potential. Fungal pathogen namely Rhizoctonia solani causing black scurf of potato and Escherichia coli (MTCC Number: 68) procured from Institute of Microbial Technology, Chandigarh were taken for present study. G. arborea was selected for fractionation, isolation and purification of bioactive compound(s). the investigations have been undertaken to standardize the procedure for macro-isolation of active principles from Gmelina arborea having antimicrobial and antioxidant activities. These investigations may be helpful in development of herbal formulations.

I. INTRODUCTION

Gmelina arborea, commonly known as Gamhar, belongs to family Verbenaceae. It is a fast growing deciduous tree, occurring naturally throughout greater parts of India at altitudes up to 1500 meters. It is used in traditional Indian medicinal system. It has also been reported to possess antimicrobial and antifungal properties[1]. There are various reports on chemical characterization of leaves, roots and fruits of G. arborea[2,3].

Gmelina arborea is locally called by different names in different languages like Gomari (Assamese); Gamari, Gambar, Gumbar (Bengali); Shewan, Sivan (Gujrati); Gamhar, Khamara, Khumbari, Sewan (Hindi); Kulimavu, Kumbulu, Kumiska, Pokki (Malayalam); Shivan, Siwan (Marathi); Bhodropornni, Gambhari, Gandhari, Kasmari, Krishnavrintaka, Sarvatobhadra, Shriparni (Sanskrit); Kumla, Kumalamaram, Kumil, Ummithekku (Tamil) and Gumartek, Gummadi, Summadi (Telugu).

1.2 Medicinal use

This woody plant has been used as cardiotonic in traditional Indian medicine. Its stem bark can be used as an antidiarrhoeal[4] and for treatment of intermittent fevers[5]. Its leaf can be used for stomach disorders[6]. It can be given orally for coughs, gonorrhoea, ulcers and can be used topically for scorpion stings and snake-bites. The crushed roasted fruit and the juice are applied externally to the itch. The roots of Gmelina are bitter, astringent, pungent in the post digestive effect and have hot potency and heavy attribute. The roots alleviate vata and kapha dosas. Its roots are used for the treatment of gonorrhea, catarrh of the bladder, rheumatism and for purification of the blood [7]. Since this plant is claimed to be useful in the treatment of rheumatism, it is

said to possess anti - inflammatory action[8]. The roots of the plant also has potent hypoglycaemic activity[9]. The roots, fruits and the leaves of gambhari have great medicinal value. The root and bark of Gmelina arborea are stomachic, galactagogue laxative and anthelmintic; improve appetite, useful in hallucination, piles, abdominal pains, burning sensations, fevers, 'tridosha' and urinary discharge[10]. Leaf paste is applied to relieve headache and juice is used as wash for ulcers[11]. Flowers are sweet, cooling, bitter, acrid and astringent. They are useful in leprosy and blood diseases. In Ayurveda, it has been observed that Gamhar fruit is acrid, sour, bitter, sweet, cooling, diuretic tonic, aphrodisiac, alternative astringent to the bowels, promote growth of hair, useful in 'vata', thirst, anaemia, leprosy, ulcers and vaginal discharge. The fruits are recommended in excessive thirst, dysuria, sexual debility in males and habitual abortion. The fruits are beneficial as anabolic in tuberculosis to hasten the healing of cavitation in the lungs. The ripened fruit is valuable in heart diseases. The cold infusion of tea prepared from gambhari fruits is extremely beneficial, mixed with honey in fever[12]. For hyperacidity, the gambhari leaves, apamarga roots and the bark-skin of salmali are mashed with cow's milk and are given orally.

Keeping in view the possibility of presence of potential constituents with medicinal and pesticidal properties, the investigations have been undertaken to standardize the procedure for macro-isolation of active principles from Gmelina arborea having antimicrobial and antioxidant activities. These investigations may be helpful in development of herbal formulations.

1.3 Objectives

To initiate the work 5 medicinal plants were identified and screened for antimicrobial activities against test organisms and antioxidant potential. Out of these plants Gmelina arborea showed potential antimicrobial as well as antioxidant potential. Fungal pathogen namely Rhizoctonia solani causing black scurf of potato and Escherichia coli (MTCC Number: 68) procured from Institute of Microbial Technology, Chandigarh were taken for present study. G. arborea was selected for fractionation, isolation and purification of bioactive compound(s).

Not much effort has been put in to develop an efficient procedure for scaling up the isolation of active compounds of gamhar (G. arborea) and to characterise its bioactive products. Attempts have also not been made to evaluate the bio efficacy of these botanicals as fungicide and antibacterial agent for the control of bacterial and fungal pathogens of crops of economic importance like potato.

Therefore, the present investigations have been undertaken to standardize the efficient procedure for macro-isolation of active principles from potential plant G. arborea as well as to develop microanalytical methods for estimation, identification of active principles and their bioefficacy against several fungi and bacteria of agricultural importance with the following objectives:

1. Screening of plants (including G. arborea) of Northern India for antimicrobial and antioxidant activity.
2. Extraction of the bark of the potential plant (G. arborea) with different solvents in bulk.
3. Standardization of procedures for macro-isolation of major active principles from the bark of G. arborea.
4. Characterization of active principle(s) with the help of modern analytical instruments such as FTIR, H-NMR, C13-NMR and mass spectra etc.

1.4 Significance:

There is no doubt that use of synthetic pesticides has led to increase in crop productivity and production. However, their continuous and indiscriminate use is responsible for the pollution of environment which has posed serious threats to mankind. It is believed that one fifth of the total applied pesticides remain in the soil, water and atmosphere in the form of residue due to persistence of the pesticides. The extensive and indiscriminate use of these xenobiotics is also responsible for a number of environmental, economic, health and other problems, such as development of resistance in pathogenic strains, resurgence of pest population, outbreak of secondary pests, pollution of environment, biomagnification, destruction of natural enemy parasites and predators, contamination of food chain and poisoning of non targeted organisms, such as fishes, wild life and human beings[13]. Synthetic pesticides may cause acute and delayed health effects in those who are exposed[14]. These effects can range from simple irritation of the skin and eyes to more severe effects such as affecting the nervous system, mimicking hormones causing reproductive problems, and also causing cancer[15]. A 2007 systematic review found that "most studies on non-Hodgkin lymphoma and leukemia showed positive associations with pesticide exposure" and thus concluded that cosmetic use of pesticides should be decreases. Strong evidence also exists for other negative outcomes from pesticide exposure including neurological, birth defects, fetal death, and neuro developmental disorder[15]. The American Medical Association recommends limiting exposure to pesticides and using safer alternatives. "Particular uncertainty exists regarding the long-term effects of low-dose pesticide exposures. Current surveillance systems are inadequate to characterize potential exposure problems related either to pesticide usage or pesticide-related illnesses. Considering these data gaps, it is prudent to limit pesticide exposures and to use the least toxic chemical pesticide or other alternatives like botanical pesticides."

Pesticide use raises a number of environmental concerns. Over 98 % of sprayed insecticides and 95 % of herbicides reach a destination other than their target species, including non-target species, air, water and soil. Pesticide drift occurs when pesticides suspended in the air as particles are carried by wind to other areas, potentially contaminating them. Pesticides are one of the causes of water pollution, and some pesticides are persistent organic pollutants and contribute to soil contamination.

In addition, pesticide use reduces biodiversity, reduces nitrogen fixation, contributes to pollinator decline, destroys habitat (especially for birds)[16] and threatens endangered species. Pests can develop a resistance to the pesticide (pesticide resistance), necessitating a new pesticide. Alternatively a greater dose of the pesticide can be used to counteract the resistance, although this will cause a worsening of the ambient pollution problem.

Use of synthetic pesticides effects economy in number of ways. Human health and environmental cost from pesticides in the United States is a total of $9.6 billion[17].

Harm	**Annual US Cost**
Public Health	$1.1 billion
Pesticide Resistance in Pest	$1.5 billion
Crop Losses Caused by Pesticides	$1.4 billion
Bird Losses due to Pesticides	$2.2 billion

Groundwater Contamination	$2.0 billion
Other Costs	$1.4 billion
Total Costs	**$9.6 billion**

Additional cost includes the registration process and the cost of purchasing the pesticides, different types of field tests and can cost between $50–70 million for a single pesticide[17]. Annually the United States spends $10 billion on pesticides[17].

Due to growing awareness of environment and public health in recent year's attention is diverted to botanical pesticides which are eco friendly, safe to use, more specific, easily biodegradable and less toxic to human beings and also beneficial to the microorganisms and non-targeted organisms. The higher plants have offered an excellent source of biologically active natural products over the centuries which help the crop plants evolve inherent defense mechanism against the pests and pathogens. These include a wide variety of secondary metabolites such as alkaloids, terpenoids, rotenoids, pyrethroids, polyacetylenes, flavanoids, unsaturated isobutyl amides, acetogenins and unusual amino acids etc[18].

There are numerous examples cited in literature of plant natural products with increasing agrochemical properties. The compounds of this type have played a major role in the development of commercial pesticides. A large number of bioactive phytochemicals are known today, which have been isolated from diverse sources and have not only provided leads to the discovery of new pesticidal molecules but have also helped in proper understanding of their modes of action.

Preliminary screening of some medicinal plants done by us indicate that Gmelina arborea possess potential antimicrobial and antioxidant activity. Hence attempts has been made for the fractionation and isolation of extracts of bark of Gmelina arborea as well as getting them separated into various components. The separated compounds are expected to be possess potential biological activity.

The result obtained will play significant role in the direction of development of herbal formulation to be used as agrochemical and/or health product.

2.1 Material and Methodology

Borosil glass apparatus and other chemicals like hexane, chloroform, methanol, etc were of Rankem Company (L.R. grade). All the solvents were distilled before use.

2.2 Instrumentation:

Melting points apparatus and electronic balance were procured from Ohaus and Labfit respectively. Flash evaporator and heating mantle were procured from Superfit and Popular traders respectively.

Infrared (IR) spectra were recorded using KBr pellets on SHIMADZU FTIR 8400S, Fourier Transform Infrared Spectrophotometer at Department of Chemistry, Lovely Professional University, Phagwara, Punjab.

UV-Vis. spectra SHIMADZU UV-1800 spectrophotometer was used to record UV-visible spectra.

^{1}H NMR and ^{13}C spectra were recorded on an AV500 FT spectrometer operating at a frequency of 500 MHz/400 MHz in DMSO/$CDCl_3$/MeOD with TMS as an internal reference.

Instruments used for antimicrobial activity are NSW-227 Vertical Autoclave, Laminar of Labfit, NSW-152 B.O.D Incubator, Labfit Bacteriological Incubator, NSW-143 hot oven and IFB microwave oven from Department of Biotechnology, Lovely Professional University.

^{1}H NMR, ^{13}C NMR, and Mass spectra were recorded at RRL Jammu, NIPER Chandigarh and Punjab University Chandigarh.

2.3 Preparation of plant extracts:

The sample of bark of G. arborea was carried to laboratory in polythene bags and allowed to dry under shade. 1.2 kg of dried plant material was grinded in mixer to pass 100 mm sieve. It was stored in airtight containers after grinding. Dry powder was extracted using soxhelet apparatus and was separately extracted with the solvents hexane, chloroform and methanol.

2.4 Separation by column Chromatography:

Separation of constituents of hexane extract of G. arborea by column chromatography:

The extract was subjected to column chromatography. The glass column (78 cm×2.5 cm) was packed with 125 g silica gel using hexane as solvent. 9.6 g of hexane extract was mixed with 11 g of silica gel to form the slurry. The dried slurry was quantitatively transferred to silica gel column. The column was eluted with 200 ml hexane followed by elution with increasing polarity solvent systems (200 ml each) i.e. hexane: chloroform :: 75:25, 50:50, 25:75, 100% chloroform; chloroform: ethyl acetate :: 75:25, 50:50, 25:75, 100% ethyl acetate; ethyl acetate: acetone :: 75:25, 50:50, 25:75 and 100% methanol was used for final washing of column. The effluents were collected as 50 ml fraction in a conical flask. They were evaporated to dryness using flash evaporator.

Separation of constituents of chloroform extract of G. arborea by column chromatography:

The extract was subjected to column chromatography. The glass column (78cm×2.5 cm) was packed with 120 g silica gel using hexane as solvent. Eight g of chloroform extract was mixed with 10 g of silica gel to form the slurry. The dried slurry was quantitatively transferred to silica gel column. The column was eluted with 250 ml hexane followed by elution with increasing polarity solvent systems (250 ml each) i.e. hexane: chloroform :: 75:25, 50:50, 25:75, 100% chloroform; chloroform: ethyl acetate :: 75:25, 50:50, 25:75, 100% ethyl acetate; ethyl acetate: acetone :: 75:25, 50:50, 25:75 and 100% methanol for final washing of column. The effluents were collected as 50 ml fraction in a conical flask. They were evaporated to dryness using flash evaporator.

Separation of constituents of methanol extract of G. arborea by column chromatography:

The glass column (78 cm×2.5 cm) was packed with 120 g silica gel. 14 g of methanol extract preabsorbed in 20 g of silica gel was quantitatively transferred to silica gel column. The column was eluted with 300 ml chloroform followed by 250 ml $CHCl_3$: CH_3OH in the ratio of 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, and 10:90. Finally the column was washed with 300ml of methanol. The fractions of 50 ml were collected in a conical flask. They were evaporated to dryness using flash evaporator.

2.5 Antioxidant activity:

Freshly prepared 60 µM solution of 1,1-diphenyl-2-picrylhydrazine (DPPH) was used as control λ max of DPPH was observed to be 517nm.

Plant extract equivalent to 4.266 mg of initial plant material was dissolved in 10 ml of absolute ethanol and then 1ml of this solution was diluted to 6 ml with ethanol to study the antioxidant potential of the respective fraction.

Two ml of DPPH solution was mixed with the same volume of the sample solution and allowed to stand for 30 minutes at room temperature. The absorbance was then measured at 517 nm. The percentage of free radical scavenging effect was calculated as follows[114]:

Scavenging effect (%) =

$$\frac{absorbance_{control} - absorbance_{sample}}{absorbance_{control}} \times 100$$

2.6 Antifungal activity:

Collection of fungal culture:

The fungal pathogen Rhizoctonia solani was procured from Central Potato Research Institute Campus, Modipuram, Meerut. The Potato dextrose agar (PDA) medium was used for culturing. The petriplate was incubated at 28±1^{o}C for three days.

Preparation of Potato Dextrose Agar:

The Potato Dextrose Agar (PDA) medium was used for culturing the fungal pathogen. 3.9 g of PDA was dissolved in 100 ml of distilled water and was heated gently so that PDA dissolves in it. Medium were autoclaved at 15 psi (121^{0}C) for 20 minutes for sterilization.

2.7 Antibacterial activity:

Collection of bacterial cultures:

The bacterial culture of Escherichia coli (MTCC No.- 68) was procured from the Institute of Microbial Technology (IMTECH), Chandigarh.

Preparation of Nutrient Agar:

The nutrient agar medium was used for culturing the bacteria E. coli. 2.8 g of nutrient agar was dissolved in 100 ml of distilled water and was heated gently so that it dissolves completely. Media were autoclaved at 15psi (121^{0}C) for 20 minutes for sterilization.

Sterilization:

(a) Glass wares:

All the glassware were washed thoroughly with liquid detergent, rinsed with chromic acid and distilled water and placed in oven at 140-160°C for 2 hrs.

(b) Media:

Glass tubes and vessels containing media were cotton plugged, covered with aluminum foil and autoclaved at 15 psi for 20 minutes at 120°C.

(c) Laminar flow safety hood:

All the processes, which include aqueous extract preparation and culture of fungi and bacteria, were performed in laminar airflow chamber which provided a sterile environment. Surface of laminar flow chamber was wiped

and cleaned with spirit. UV light was switched on for 20 min. and all non living material as glassware etc. were exposed to it. This was followed by allowing fresh air to pass through it.

(d) Preparation of slants and sub culturing:

Pure fungal culture growing on petriplates containing PDA was used for preparation of slant cultures which was used in subsequent culturing of respective fungi.

Single bacterial colony growing on petriplates containing nutrient agar was picked to inoculate the slant, which was used for subsequent culturing of respective bacteria.

The PDA containing petriplates were streaked by taking a loopful inoculum from slants of respective fungi in laminar flow safety hood and incubated under humidified conditions at 28±1 °C in BOD incubator.

For bacterial culture Nutrient Agar containing petriplates were streaked in laminar flow safety hood by taking inoculum from slants of respective bacteria and incubated in incubator at 37 ^{0}C. Bacterial cultures in liquid media were used for inoculation to plate containing nutrient agar to study the effect of plant extract on growth of bacteria.

2.8 Screening of extracts for fungicidal activity:

The fungal pathogen Rhizoctonia Solani that causes the disease in potatoes was selected for present investigations. Initially, paper disc method was used for screening anti-fungal activity of plant extracts against test organism[19]. This method was based on diffusion capacity of test chemical(s) through agar medium. Fungal plug were placed at the center of assay plate containing sterilized PDA and allowed to grow. After circular growth of about 2-3 cm diameter four sterilized paper disc (two loaded with 20 µl plant extract equivalent to 14 mg of initial plant material and two with same amount of pure solvent) were placed at equal distance from center in order to see the effect of plant extract on the growth of fungal pathogen. Plates were incubated at 28±1°C. Inhibition zones were measured after three days of incubation depending upon the growth of pathogen. Dumb bell shaped growth of fungus was observed in case of extracts containing growth inhibitory component(s). Subsequently food poisoning technique[20] was used to find percent inhibition of alcoholic plant extracts showing potential activity against certain test organisms. For this purpose 50µl of methanol extract equivalent to 35 mg initial plant material was spread to each petridish after pouring the sterilized medium, while in control treatment equal amount of solvent was added. The fungal plug was placed at the centre of petridish and the petriplates were placed at 28±1°C temperature. Growth of fungus was recorded after three days. The percent inhibition was calculated using the formula of Vincent[21]:

$$\text{Inhibition (\%)} = (C\text{-}T)/C \times 100$$

Where C is the growth in control in mm and T is growth in treatment in mm.

The correlation coefficient (r) and coefficient of determination (r^2) was calculated by using the formula[22]:

$$r = \frac{n\sum xy - \sum x \sum y}{\sqrt{\{[n\sum x^2 - (\sum x)^2][n\sum y^2 - (\sum y)^2]\}}}$$

The significance test (t test) was calculated by the formula:

$$t = r\frac{\sqrt{(n-2)}}{\sqrt{1-r^2}}$$

Screening of extracts for antibacterial activity:

Molten nutrient agar medium was poured in sterilized petriplates and allowed to come at room temperature. One hundred µl of bacterial culture in nutrient agar was added to petriplates. After solidification one sterilized paper disc loaded with 20 µl (equivalent to 7.6 mg of intial plant material) plant extract and another with pure solvent were placed in petriplates and kept in incubator at 37ºC. Inhibition zones were measured after 36 hrs. of incubation[20].

2.9 Thin Layer Chromatography of Bioactive Fractions:

Bioactive fractions of hexane and methanol extracts of G. arborea were analysed using thin layer chromatography. The TLC plates were washed properly and then cleaned with acetone and dried in oven. They were then placed on a platform. The slurry of silica gel G was made in distilled water. It was then sprayed on the plates by use of applicator. The uniform distribution of silica occurred on the plates. These plates were then placed in oven for 4-5 hrs. for proper drying. Prior to use these plates were activated in oven at 120ºC temperature for one hour.

Proper solvent systems were selected for isolation of the components of different fractions collected by column chromatography. I_2 or UV light was used for visualization.

Melting point, I.R., N.M.R., M.S. and UV-VIS spectra of purified compounds were recorded and attempts were made to identify these components.

3.1 Result and Discussion

.In present work some medicinal plant species namely Prunella vulgaris, Emblica officinalis, Withania somnifera, Berginia lingulata, collected from Northern India have been screened for their antimicrobial and antioxidant activity. Bark extract of Gmelina arborea shows potential antimicrobial activity against both the test organisms (Rhizoctonia solani and E. coli) taken for present study. All the extracts of Gmelina arborea showed potential antioxidant activity. Encouraged by these results and keeping in view the possibility of presence of already reported chemical substances[23], an attempt has been made for fractionation and isolation of the extract (extraction by hexane, chloroform and methanol) from bark of Gmelina arborea as well as for getting them separated into various components, purification of components and subjecting them for various methods of identification. The chemical constituents separated from the bark extract of Gmelina arborea are expected to possess potential antimicrobial and/or antioxidant activity.

Besides the reported chemical constituents other compounds are also expected to be present in these extracts. The isolation, purification and identification is important from the view point of developing herbal formulations for management of micro-organisms taken for the present study as well as developing antioxidant diet supplements. Therefore the separated compounds will be subjected to the studies related to their specific antimicrobial properties.

Fractionation and isolation of extracts of bark of Gmelina arborea:

The hexane, chloroform and methanol extracts of dried powder of bark of Gmelina arborea are prepared. These extracts were subjected to gradient elution column chromatography using the following parameters:

Column length = 78.5 cm,

Column diameter = 2.5 cm.

Adsorbent – silica gel H (60-120) mesh size.

Solvent system used for elution in increasing order of polarity for hexane, chloroform and methanol extracts respectively.

A total of 42, 35 and 20 fractions of 50 ml each were collected in case of hexane, chloroform and methanol extracts respectively. These fractions were subjected to thin layer chromatography. Constituent with Rf value 0.4 eluted with solvents hexane and chloroform in the ratio of 9 : 1 (isolated from fraction 3 GA-1); Rf 0.55 eluted with solvent hexane and chloroform in the ratio of 7 : 3 (isolated from fraction 12 GA-2) and the constituent with Rf value 0.6 eluted with solvent system hexane and chloroform in the ratio of 6 : 4 (isolated from fraction 14 GA-3) could be only collected in sufficient quantity to carry out spectral studies. The chemical constituent with Rf value 0.93 by solvent system chloroform : acetic acid : water in the ratio of 50 : 45 : 5 (isolated from fraction 14 GAM-1) of methanol extract only could be collected in sufficient quantity to carry out IR, NMR, Mass and UV visible spectra of the compound for the characterization purpose.

3.2 Identification of compound GAM-1 extracted from methanol extract:

Melting point: This compound was recrystalized with ethanol, its melting point was recorded to be 305-309 ^{0}C, while the value of melting point reported in literature is 310 – 316 ^{0}C.

Chemical test: It gives Shinoda test to form yellow or crimson red colour when it mix with magnesium ribbon and concentrated hydrochloric acid.

UV Visible spectrum: The UV of this component (recorded in methanol) showed λ_{max}. at 380 nm and 250 nm signifying the $\pi - \pi^*$ transitions and $\sigma \rightarrow \sigma^*$ transitions respectively. The data is in good agreement with the values reported for the compound.

IR spectrum: The IR spectrum in KBr of this compound show bands at 1658 cm^{-1} (due to C-O stretching); 1608 cm^{-1} 1558 cm^{-1} 1516 cm^{-1} and 1454 cm^{-1} (due to C=C aromatic ring stretch); 1365 cm^{-1} (due to plane O-H bend of phenols); 1319 cm^{-1} (due to out of plane bending of C-H bond in aromatic hydrocarbons); 1255 cm^{-1} (C-O stretch of aryl ether); 1207 cm^{-1} (C-O stretch of phenol); 1168 cm^{-1} (C-C, O-C stretch and bending in ketone); 935 cm^{-1} 821 cm^{-1} 796 cm^{-1} and 696 cm^{-1} (out of plane C-H bending of aromatic hydrocarbons).

H-N.M.R. spectrum: The proton NMR of this compound recorded insolvent MeOD . the δ and J value obtained are given in table 4.1. these values are in good agreement with the reported values[24].

Table 4.1: H- NMR data for GAM-1 isolated from methanol extract

Position	δ value	J value
H-6	5.9708	
H-8	6.1767	
H-5'	6.68 (d,d)	12.2, 3.9
H-6'	7.4(d)	8.32
H-2'	7.52	
Aromatic C-OH, attached to C-5, 7, 3', 4'	4.67	

C-13 N.M.R. spectrum: C-13 spectrum of this compound recorded in MeOD is given in the table 4.2

Table 4.2: ^{13}C NMR data (75 MHz) for GAM-1 isolated from methanol extract

Position	Signal (δ)	δ Calculated[120]
2	156.80	160.0
3	135.81	136.6
4	175.90	178.3
5	97.81	98.3
6	164.14	166.4
7	92.99	98.0
8	147.35	160.5
9	161.08	160.01
10	103.10	105.5
1'	122.73	124.4
2'	114.58	113.6
3'	144.80	147.2
4'	146.57	146.5
5'	114.81	117.2
6'	120.26	120.4

Mass spectrum: The ESIMS exhibited $[M+H]^+$ and [M+Na] peaks at m/z 303 and 325 respectively, corresponding to molecular formula $C_{15}H_{10}O_7$, m/z signal corresponding to [M-CO], [M-CO_2] and $C_7H_8O_2$ were observed at 273, 257 and 151 respectively.

The compound is characterized as biflavonol composed of two benzene rings link with a heterocyclic pyron ring (aromatic, trimeric, heterocyclic). It is insoluble in water and is characterized as 2-(3,4 di-hydroxy phenyl)-3,5,7 trihydroxy 4-H-1-benzopyran-4-one.

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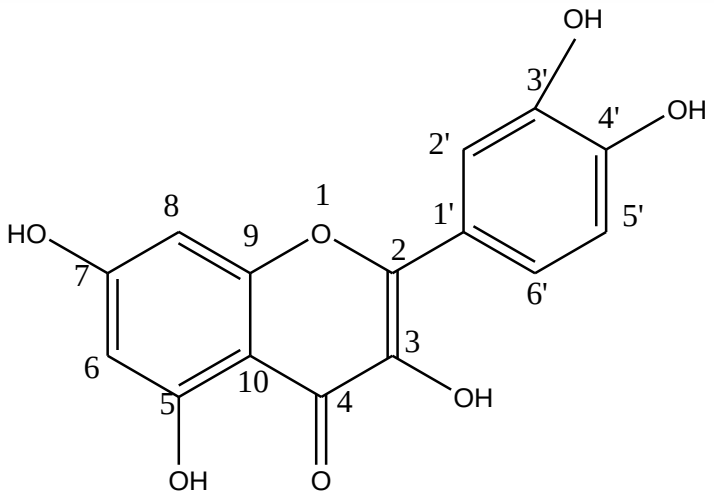

3.3 Spectral studies of GA-1 isolated from hexane extract of bark of Gmelina arborea:

1. Melting point: Its melting point was recorded to be 80-83 ^{0}C.

2. Chemical test: It shows positive test for terpenoids.

3. UV-visible spectrum: The UV spectrum of this compound was recorded in ethanol and λ_{max} has been observed at 195 nm.

4. IR spectral studies: The IR spectrum of GA-1 suggests the presence of different functional groups ranging from O-H stretching of hydroxyl group (3423.76 cm^{-1}), C-H stretching of alkyl group (2962.76 cm^{-1}), C-H bending (1462.09 cm^{-1}), C=C stretching of aromatic ring (1670 - 1420cm^{-1}), C-O bending (1379.15 – 1041.28 cm^{-1}).

5. H-NMR spectrum: Proton NMR spectrum was recorded in $CDCl_3$ suggests presence of terpene moity, (NMR signal in the range of 0.84 – 3.56 ppm), an aromatic ring (δ = 7.9031 ppm).

6. C-13 NMR spectrum: ^{13}C NMR spectrum was recorded in $CDCl_3$, the signals have been observed at δ = 13.11, 21.68, 28.35, 28.68 and 30.91 ppm. The observations support the presence of terpene moity.

It is required to further purify the compound for assigning the structure.

3.4 Spectral studies of GA-2 isolated from hexane extract of bark of Gmelina arborea:

1. **Melting point:** Its melting point was recorded to be 109 – 112 ^{0}C.

2. Chemical test: It shows positive test for terpene.

3. UV-visible spectrum: UV-visible spectrum of this compound was recorded in ethanol, two maxima have been observed at 212 and229 nm.

4. IR spectral studies: IR spectrum of GA-2 in KBr shows bands at 3406.40 cm^{-1} (due to O-H stretching of hydroxyl group); 2958.90 cm^{-1}, 2918.40 cm^{-1} and 2848.96 cm^{-1} (due to C-H stretching of CH_2 and CH_3); 1464.02 cm^{-1}, 1379.15 cm^{-1} (due to C-H bending); 1261.49 cm^{-1} and 1168.49 cm^{-1} (due to C-O stretching).

5. H-NMR spectrum: Proton NMR spectrum was recorded in $CDCl_3$ nmr signal have been observed in the range of 0.6 – 5.27 ppm, indicating the presence of terpene moity.

6. C-13 NMR spectrum: ^{13}C NMR spectrum was recorded in $CDCl_3$, the signals have been observed in the range of δ = 13.10 to 38.83 ppm. The data support the presence of terpene moity.

It is required to further purify the compound for assigning the structure.

3.5 Spectral studies of GA-3 isolated from hexane extract of bark of Gmelina arborea:

1. Melting point: Its melting point was recorded to be 55-60 ^{0}C.

2. UV-visible spectrum: The UV spectrum of this compound was recorded in ethanol and λ_{max} has been observed at 275 nm.

3. IR spectral studies: The IR spectrum of GA-3 recorded in KBr shows bands at 2918.40 cm^{-1}, 2848.96 cm^{-1} (due to the C-H stretching of alkyl group); 1735.99 cm^{-1} (due to C=O stretching); 1464.02 cm^{-1}, 1377.22 cm^{-1} (C-H bending); 1172.76 cm^{-1}, 1095.60 cm^{-1} and 1028.09 cm^{-1} (due to C-O stretching).

4. H-NMR spectrum: Proton NMR signals were observed in the range of 0.6 – 5.5 ppm .

5. C-13 NMR spectrum: ^{13}C NMR spectrum was recorded in $CDCl_3$, the signals have been observed in the range of 11.86 to 31.94 ppm supporting the presence of terpene moity.

3.6 Studies on the biological properties of the separated fractions:

Evaluation of antifungal activity against fungal pathogen of economically important crop potato:

The plant botanicals are known to possess medicinal properties and biocidal activity against microbial and other pests and pathogens. Botanical pesticides are biodegradable target specific and less toxic to non targeted organisms. In the present study, antifungal activity of G. arborea has been evaluated against Rhizoctonia solani causing black scurf of potato.

Hexane and chloroform fractions of bark of Gmelina arborea have not shown antifungal activity against R. solani causing black scurf of potato. However methanol extract has been observed to exhibit potential antifungal activity against R. solani. The antifungal potential of fractions isolated from methanol extract has been reported in figure 4.1 and 4.2. Fraction 5 has been found to be most potent against R. solani with an inhibition zone of 12 mm followed by fraction 6, 17 and 18 causing inhibition zone of 10 mm with 71.42 to 67.32 %.

The value of correlation coefficient (r) and coefficient of determination (r^2) for antifungal activity of methanol extract have been calculated to be 0.97287 and 0.94647 respectively. For observation related to inhibitory effect of fractions isolated from methanol extract of bark of Gmelina arborea against Rhizoctonia solani. The value of r^2 shows that 94.647 % inhibition was caused by fractions of methanol extract and rest 5.353% may be attributed to other unknown factors.

The calculations related to the significance test (t test) revealed that the value of "t" 17.844 is much higher than the critical value (3.850) noted from "t" distribution table for degree of freedom 20 at 0.05% significance level. This suggests that there are less than 0.05% chances of error in drawing the above conclusions.

Evaluation of antibacterial activity against Escherichia coli causing various infectious diseases:

Antibacterial activities of fractions isolated from hexane extract have been reported . Inhibition zone of 5 – 18 mm have been observed for fractions isolated from hexane extract of G. arborea. Maximum inhibition zone has been recorded for fraction number 6, 19, 22 and 28 with zone size of 18 mm each, followed by fraction number 7, 9, 12, 20, 29, 30 and 31with zone size of 16 mm. Other fractions have also been found to possess potential activity against E. coli (figure 4.3).

Chloroform extract and fractions separated from it have been observed to possess maximum antibacterial potential among hexane, chloroform and methanol extract. The activity data has been reported in figure 4.4

Fraction 21 has exhibit maximum antibacterial potential with inhibition zone of 27 mm followed by fraction 12 with zone size of 26 mm. Twenty two fractions out of total of 35 fractions have been exhibited significant antibacterial activity against E. coli with zone size greater than or equal to 15 mm.

Antimicrobial activity of fractions of methanol extract has been reported in table 4.7. Out of total of 20 fractions, 18 fractions have shown potential activity against E. coli with zone size greater than 17 mm (Figure 4.5). Fraction 14 eluted with solvent chloroform: methanol:: 4 : 6 has been found to possess maximum bactericidal potential with inhibition zone of 24 mm.

Evaluation of antioxidant activity:

Antioxidant activities of extracts and fractions isolated have been evaluated to explore the possibility of role of G. arborea in drug development for prevention and cure of various diseases caused by oxidative stress like cancer. All the extracts of G. arborea have been found to possess excellent antioxidant activity potential. Antioxidant activity have been evaluated by radical scavenging technique using DPPH. Antioxidant potential of fractions separated from hexane, chloroform and methanol has been reported . Out of total of 42 fractions isolated from hexane extract, 37 fractions have shown significant antioxidant activity greater than 50 % (Figure 4.6). Fraction 22 eluted with solvent system chloroform: ethyl acetate:: 7.5 : 2.5 possess maximum of 78 % antioxidant potential.

Twenty three fractions out of a total of 35 fractions separated from chloroform extract have been found to possess greater than 50 % antioxidant potential (Figure 4.7). Fraction 26 eluted with ethyl acetate (100 %) have been found to possess 75.7 % activity followed by fraction 25 with 74.5 % of antioxidant potential.

Fractions isolated from methanol extract have been found to possess maximum antioxidant potential. The data of activity has been reported in table 4.9. fifteen fraction out of total of 20 fractions have been found to possess greater than 50 % antioxidant activity (Figure 4.8). Three fractions (fraction number 17, 19 and 20) have been found to possess greater than 80 % activity. Fraction 19 has exhibited 88.77 % antioxidant potential.

The results of the work reported in detail are summarized and discussed below:

1. Antifungal, antibacterial and antioxidant activities of different extracts of G. arborea have been evaluated. The comparative studies made in this chapter reveal the interesting observation that the chloroform extract is better in antibacterial potential in comparison to the hexane and methanol extract of G. arborea, while methanol extract exhibit better fungicidal and antioxidant potential. On the basis of these observations we can conclude that bark of Gmelina arborea possess a wide spectrum of biological active chemical constituents. These findings will be helpful in exploring the potential role of G. arborea in development of herbal formulation to be used as health care products and agrochemicals

2. Keeping in view the possibility of existence of many more significant chemical constituents in addition to those reported, so far in the bark of Gmelina arborea the hexane, chloroform and methanol extracts of dried bark was subjected to activity guided fractionation, isolation, purification and identification. The techniques employed for this proposed work includes column chromatography, thin layer chromatography, recrystallization, melting point determination, UV- visible spectra, IR, H- NMR, C-13 NMR and Mass spectroscopy . One of three active principles could be isolated from methanol and hexane extracts respectively. Out of these GAM-1 isolated from fraction 14 of methanol extract could be purified and identified as 2-(3,4 di-

hydroxy phenyl)-3,5,7 trihydroxy 4-H-1-benzopyran-4-one. However active principles (GA-1, GA-2 and GA-3) isolated from hexane extract could not be identified due to presence of some impurities.

3. GAM-1 with molecular weight 302 has been characterized as the major constituent of methanol extract of bark of G. arborea (4.1-4.4). GA-1, GA-2 and GA-3 isolated from hexane extract shows positive test for terpenes. NMR spectra of these compounds also support the presence of terpene moity. These compounds could not be characterized due to presence of some impurities. Further purification of these compounds with the help of preparatory HPLC is required to purify and characterize these compounds.

4. The results on specific antimicrobial activities of hexane, chloroform and methanol extracts of bark of Gmelina arborea against E. coli (MTCC 68) and R. solani (a fungal pathogen causing black scurf of potato) have already been discuss . Fraction 14 of methanol extract that has been found to contain 2-(3,4 di-hydroxy phenyl)-3,5,7 trihydroxy 4-H-1-benzopyran-4-one shows fungicidal as well as antibacterial potential. It is evident from the data reported in table 4.11 that 2-(3,4 di-hydroxy phenyl)-3,5,7 trihydroxy 4-H-1-benzopyran-4-one is the main principle possessing antibacterial and antifungal activity. Other constituents present in fraction 14 of methanol extract of G. arborea may possess antimicrobial activity or these may work as synergist with active principle GAM-1. Fraction 3, 12 and 14 of hexane extract of G. arborea and GA-1, GA-2 and GA-3 isolated from these fractions do not possess fungicidal potential against R. solani. GA-1 and GA-2 are active principles of fraction 3 and 12 respectively possessing antibacterial activity against E. coli. Other components present in fraction 3 and 12 may possess antibacterial activity against the test pathogen or may act as synergist.

5. Comparison of antioxidant activity of fraction number 14, 3, 12 and 14, the constituents isolated from these fractions, GAM-1, GA-1, GA-2 and GA-3 has been reported. A perusal of these data reveals that the compounds GA-1 and GA-2 are less potent in comparison to the respective fraction from which these were separated. It means that these chemical compounds, when used alone, are not as effective as in the case when they are applied in combination with certain active principles present in the same fraction of the hexane extract of bark of G. arborea. Hence it can safely be assumed that compound GA-1 and GA-2 possesses the synergistic effect in the same way as reported by other workers in other plants. Further work in this direction may be planned to develop synergistic formulations.

Antioxidant activity of GA-3 has been found to be equal to the activity of the fraction from which it has been isolated. It may be noted that the potential is improved remarkably on using GAM-1 leading to the assertion that the antioxidant potential of respective fractions is primarily due to the constituents GAM-1 and GA-3 that have been separated by me. However more studies are required in this direction so that the synergism / biopotentiation may be achieved.

The results described here may be helpful in developing plant based herbal formulation(s) to be used as health care product(s) and agrochemicals.

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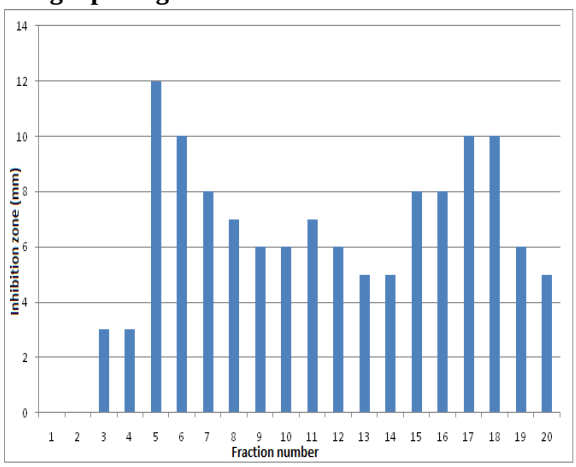
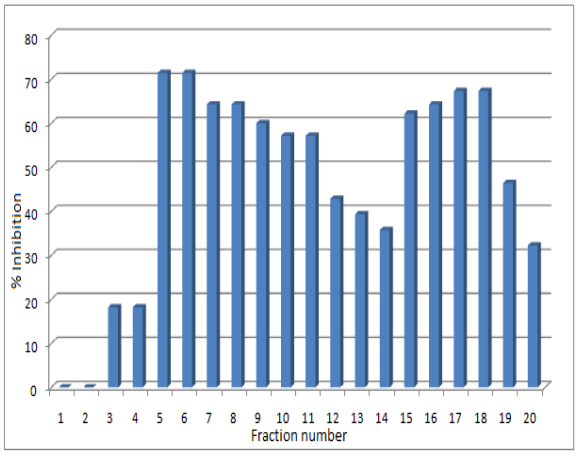
Figure 4.1 & 4.2:Antifungal activity of fractions isolated from methanol extract (paper disc method and food poisoning method respectively)

Fungal pathogen: Rhizoctonia solani

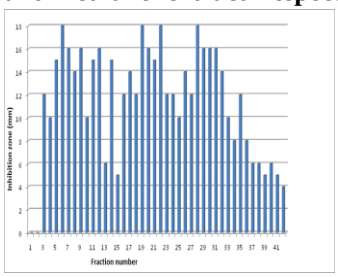


Fig(4.1)

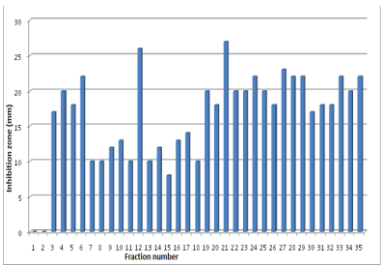


fig(4.2)

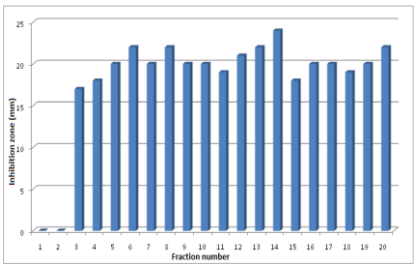
Figure 4.3 ,4.4 &4.5: Antibacterial activity of fractions isolated from hexane extract, chloroform extract and methanol extract respectively against Escherichia coli



Fig(4.3)

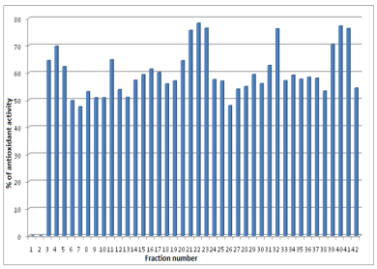


fig(4.4)

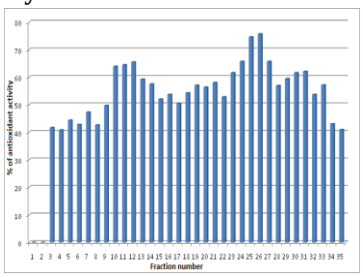


fig(4.5)

Figure 4.6 ,4.7 & 4.8: Antioxidant activity of fractions isolated from hexane extract and choloroform extract and methanol extract respectively.



Fig(4.6)



fig(4.7)

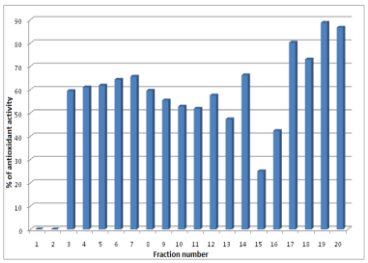


fig (4.8)

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