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Antifungal, antioxidant and DNA protection potential of *Grewia asiatica* L. leaves acetone extract

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Abstract
Grewia asitica Linn. is folk medicine as its all plant parts are used for health benefits and disease treatment. The leaves are thought to possess antimicrobial properties and hence are used for wound and cut treatment as well as instant relieve from irritation and painful rashes. Fruits, stem bark and root bark are widely applied for treatment of ailments like indigestion, asthma, spermatorrhoea, heart, blood and liver disorders, rheumatism and urinary tract problems. *Grewia asitica* Linn. leaves were dried and phytochemicals were extracted in acetone using soxhlet. The crude extract thus obtained was analyzed for its total phenolic and total flavonoid content. GALAE was then investigated for antifungal, antioxidant. The findings demonstrated strong antifungal activity of the *Grewia asitica* Linn. leaves acetone extract (GALAE) against highly pathogenic fungi including *Aspergillus* spp., *Candida* spp., *Microsporum* spp. and *Trichophyton* spp. which belongs to risk group two organisms. The antioxidant activity of GALAE as evaluated by DPPH method suggested very high content of free radical scavengers that were effectively protecting DNA damage caused by free radicals generated by Fenton's reagent. The results of present study indicated that acetone extract of *G. asiatica* Linn. is having strong antifungal and antioxidant activity, which can be by virtue of the presence of phenolics, flavonoids and saponins. Hence it can be further explored for its possible application for treatment of fungal diseases like dermatophytosis, aspergillosis, candidiasis, and zygomycosis, as well as metabolic disorders where reactive oxygen species have major contributions in pathogenesis of diseases like diabetes, stroke, rheumatoid arthritis, cardiovascular diseases and cancer and reduce ageing effect.

Keywords: *Grewia asiatica* L., antifungal activity, antioxidant, DPPH, DNA protection activity

Introduction
The global market of plant derived products is valued at $83 billion US and still growing. Furthermore, ballpark figure has reached to 25% of modern drugs with 60% of antitumor drug being naturally derived. As per World Health Organization (WHO) estimate, between 65% and 80% population of the developing country dwellers rely on medicinal plants as remedies (Gomathi *et al*., 2012) [12]. Of the 300,000 plant species existing on earth, only 15% has been explored for their pharmacological potential. This is the urgent call for demonstrating the efficacy and drift for medicinal plants to be used as drug potential candidate. Indian medicinal plants are the essence of Ayurveda and Ayurvedic treatments. Various medicinal plants investigated by dry weight loss method have been found to be effective against human dermatophytes under standard laboratory conditions (Singh *et al.*, 2010) [40]. In yet another study carried out by Singh *et al*. (2008) [38], 32 plant species were reported to have therapeutic values for skin disease treatments along with candidiasis. Masih *et al*. (2014) [27] reported that leaves extract of *Pongamia pinnata, Curcuma longa, Nerium indicum and Calotropis procera* were effective against plant pathogenic fungi through well diffusion method. In the Indian systems of medicine, most practitioners particularize and give away their own prescription hence this requires proper certification and delvelope fruitage diverse bioactive compounds (Ghosh *et al*., 2003) [11]. The plant in present study i.e. *Grewia asiatica* Linn. is a member of genus Grewia of Tiliaceae family that comprises a total of 11 genera. Various members of Tilaiceae family have medicinal importance like flower of *Tilia flos* Linn. is recommended for treatment of cough, cold and flu (Füleky, G. 2016) [10], leaves and fruits of *Microcos paniculata* Linn. are administered for relief from indigestion, typhoid, dysentery mouth ulcer, eczema, small pox etc., crushed leaves of *Corchorus olitorus* Linn. are used for treatment of asthma while leaves of *Triumfetta cordifolia* A Rich. are used to prevent vomiting (Macfoy, C. 2013) [26]. Members of *Grewia* genus are shrubs and trees. More than 35 species of this genus have their distribution in Indian subcontinent in the warmer region of Punjab, Western Himalaya up to 1000 m, Garhwal, Sikikim, north Bengal, Bihar, Chota Nagpur, Orissa, Eastern Asam, Gujarat, Konkan, Deccan and South India, forests of Dun and Saharanpur

(Nadkarni, K.M. 1996; Joshi *et al*., 2009) [29, 20]. Some members of *Grewia* are well known for their therapeutic application. *Grewia asiatica* Linn. is reported in Ayurveda and Unani medicine therapy to have various medicinal values. Leaves, fruits and seeds are widely used for curing of spleen disease, alcoholism and cough. The fruits of *G. asiatica* Linn. locally called as phalsa, is well-known for its nutritional and therapeutic element. Sharbat-e-phaalsa is recommended in unani therapy as cardiac tonic. The bark infusion of *Grewia asiatica* Linn is demulcent while leaves are used in pustular eruptions (Khare C.P. 2004) [23]. The phalsa plant has been used by many tribes of India for medicinal purposes. The Goanese use roots of *Grewia asiatica* Linn as substitute of althea for treatment of rheumatism. The tribel communities from Singrauli, M.P., India use leaf paste for postural eruptions and their fruits as astringent and cooling (Chauhan R.S. 2017) [5]. Despite having so much medicinal importance, Phalsa plant has remain neglected due to lack of experimental evidences and literature (Zia-Ul-Haq *et al*., 2013) [46].

2. Materials and Methods

2.1. Plant material

The fresh and healthy leaves of plant were collected from various sites of Gwalior (M.P.) in the month of January–February 2015. Plant material was identified with the help of local community and was authenticated by Dr. Harison Masih, Assistant professor at Department of Industrial Microbiology, JIBB, SHUATS, Allahabad.

2.2. Extract preparation

Freshly collected plant leaves were surface sterilized using 0.1% $HgCl_2$ followed by repeated washing with sterile phosphate buffer saline (pH 7.2) and distilled water. Plant leaves were than dried at 50°C and crushed with the aid of a mechanical grinder to powdered form. Twenty five grams (25 g) of dried plant powder was packed in Whatman filter paper no.1 and was extracted in a soxhlet apparatus using 250 mL of acetone (Fong H.H.S., 1973) [9]. The extracts were concentrated on rotavapour (NAC-2001 NS) and then dried in lyophilizer under reduced pressure to obtain dried solid residue. The dried extracts were stored in refrigerator at 4°C.

2.3. Fungal culture isolation and identification

Standard cultures of fungal species used in present study were made available by dept. of Industrial Microbiology, SHUATS, Allahabad. Along with this, fungal cultures were isolated from human clinical samples as well as soil samples collected from local hospitals of Gwalior and Allahabad. Accordingly, the cultures were coded as Boston hostel sample (BHK), SHUATS standard culture (SH), Kamlaraja hospital sample (KHK), Mangal Hospital sample (MHK), Cancer hospital sample (CHK) and environmental collect (EC). The cultures were identified based on microscopic and morphological characteristics as well as pigmentation pattern. The results were cross verified with literature available from National Mycology reference centre, Adelaied, South Australia (Ellis *et al*., 2007) [7].

2.4. Antifungal activity

2.4.1. Agar well diffusion method

The *in vitro* antimicrobial susceptibility test was performed using agar well diffusion method (Perez *et al*., 1990) [32] using 100 µL of prior activated cultures in Sabouraud's dextrose broth. Wells were punched using sterile cork borer of 7 mm and loaded aseptically with 70 µL of GLAE from stock of 500 mg/mL concentration dissolved in DMSO. Dimethyl sulphoxide (70 µL) was used as control. The plates were left undisturbed at room temperature in order to allow diffusion of extracts in the agar medium (Esimone *et al*., 1998) [8]. The plates were then incubated at 37^0C for 24-48 hours and observed for zones of inhibition around the wells. The diameters of zones of inhibition were measured in millimeters (mm). The degree of antifungal activity was calculated by taking mean of the values obtained for the zone of inhibition on each of the replicate agar plates (Jain *et al*., 2012) [18].

2.4.2. Determination of minimum inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The determination of MIC for crude extracts was carried out using the test tube dilution method (Cruckshank *et al*., 1975) [6]. In each tube, 100 µL of each extract dilution (10 mg/mL, 35 mg/mL, 70 mg/mL, 140 mg/mL, and 280 mg/mL) was mixed with 100 µL of the fungal spore suspension (2×10^6 spores mL^{-1} in fresh PDB). The tubes were incubated for 48 to 72 hrs at 37°C with intermittent observation for mycelia. All experiments were done in triplicate. The MFC was determined by spot culturing from each tube on fresh solid medium followed by incubation at 37°C for 18-24 hrs. The lowest concentration of MIC tubes with no visible fungal growth on solid medium was regarded as MFC.

2.4.3. Total Phenolic compounds

Folin-Ciocalteu reagent was used for total soluble phenolics estimation, gallic acid as a standard. To the 1.0 mL of extract solution in a test tube, 0.2 mL of Folin Ciocalteu reagent (1:2 dilutions) was added. After 20 minutes of incubation, 2.0 mL of water and 1.0 mL of sodium carbonate (15%) were added. Above reaction mix was allowed to stand for 30 minutes and then absorbance was measured at 765 nm. The concentration of total Phenolic component in the sample was determined as microgram of gallic acid equivalent (GAE) (Panovska *et al*., 2005) [30].

2.4.4. Total flavonoid content

Aluminium nitrate was used to determine total soluble flavonoid of the extract, quercetin taken as standard. To 1 mL of 80 % ethanol plant extracts were added followed by aliquot of 0.5 mL, 0.1 mL of 10 % aluminium nitrate, 4.3 mL of 80 % ethanol and 0.1 mL of 1 M potassium acetate. Ion mixture is allowed to stand for 40 min in room temperature followed by absorbance measured at 415 nm (Cameron *et al*., 1943; Govindarajan *et al*., 2003) [4, 14].

2.5. *In vitro* antioxidant activity by DPPH method

In the presence of DPPH stable radical the hydrogen donating ability of extracts was evaluated. One millilitre of DPPH ethanol solution (0.3 mM) was added to 2.5 mL of sample solution of constant concentration and allowed to stand at room temperature. After 30 minutes of incubtion the absorbance values were taken at 517 nm. Plant extract solution with ethanol was used as a blank while DPPH solution (1.0 mL, 0.3 mM) dissolved in ethanol act as negative control. Ascorbic acid solutions were used as positive controls (Gomes *et al*., 2001) [13].

2.7. DNA protection activity

Oxidative DNA damage was induced by hydroxyl radicals generated by Fenton reaction. The reaction mixture containing 25 mg of Salmon sperm DNA in 20.0 mM phosphate buffer

saline (pH 7.4), 15µg/mL of various plant extracts and DNA were kept in incubation for 15 minutes at room temperature. DNA oxidation was induced by adding 20 mM ferric nitrate and 100 mM ascorbic acid. The above reaction mixture was incubated for 1 hour at 37°C. DNA loading dye was used to terminate the reaction. The mixture was subjected to gel electrophoresis and visualized under UV trans-illuminator (Khan *et al*., 2012) [21].

2.9. Statistical Analysis
Data recorded during the course of investigation were subjected to appropriate statistical analysis using GraphPad Prism 5 software.

3. Result and discussion
Human diseases caused by fungal pathogens pose a significant burden on public health affecting over 1 billion people worldwide. Invasive fungal pathogens of blood, lungs and other organs contribute serious risk to millions of immune-compromised subjects including HIV/AID and those receiving chemotherapy. Effectiveness of available antifungal drugs has diminished due to their extensive and inappropriate use leading to emergence of drug resistant fungal pathogen strains. Present generation of drugs also poses harmful side effects, negative interactions with other drugs and inability to be administered orally, thus driving towards new findings and production of improved and more effective antifungal drugs. Medicinal plants are armory for safe and effective medicines (Tiwari S., 2008) [45]. As per World Health Organization (WHO) records, more than 75% of the world's population rely on the traditional medicine (Kumar *et al*., 2012) [24] either in the form of plant extracts or pure compounds for their treatment (Parekh, 2007) [31]. Various studies have been conducted worldwide to describe the antimicrobial activities of different plant extracts (Almagboul *et al*., 1985[1]; Artizzu *et al*., 1995 [2]; Bonjar *et al*., 2004 [3]; Ikram M and Inamul H, 1984 [16]; Islam *et al*., 2008 [17]; Shapoval *et al*., 1994 [33]; Somchit *et al*., 2003 [42]; Sousa *et al*., 1991 [43]) against bacterial as well as fungal pathogens.

In the present study, *Grewia asitica* commonly known as phalsa, was selected for studying antifungal, antioxidant, DNA damage protection ability and cytotoxic activity. These studies were performed using leaves acetone extract of *G. asitica*. Pathogenic fungal species isolated from human clinical samples and soil samples collected from local hospitals of Gwalior and Allahabad, were use to evaluate antifungal activity of GALAE. The isolated fungal cultures were characterized and identified on the basis of morphological and microscopic examination, results of which are presented in Table 1. The effectiveness of GALAE against 10 fungal cultures was evaluated using agar well diffusion method. Maximum inhibition zone of 32 mm was obtained against SH 2 i.e. *Aspergillus fumigatus* followed by 28 mm against SH 8 i.e. *Candida glabrata*. The cultures SH 2 and SH 8 were used as standard for antifungal study. Among 8 fungal isolates, GALAE exhibited maximum inhibitory activity against CHK 2, CHK 5 and MHK 5. MIC and MFC was also estimated to quantitatively measure the antifungal potential of the extract**.** GALAE inhibited growth of most of fungal pathogens at the concentration of 10mg/mL while it showed its fungicidal activity for most of the cultures at 35 mg/mL concentration. The results of zone of inhibition, MIC and MFC are presented in Table 1. Simillar results of antifungal activity of different plant extracts have been reported earlier by various workers. Masih *et al*. (2015) [28] observed the antifungal activity of *Ocimum sanctum* extracts against sinusitis fungal pathogens. Jasim *et al*. (2013) [19] reported the antifungal properties of Garlic, Fenugreek, Ginger plant extracts against *Candida* species. Singh *et al*. (2009) [39] reported that the medicinal plant extracts showed therapeutic values against dermatophytic pathogens as growth inhibition by well diffusion process. Various plant parts are known to contain different secondary metabolites like phenols, polyphenols, flavonoids, alkaloids, tannins, terpenoids, saponins, essential oils, flavones and glycosides to name a few. These secondary metabolites have immense therapeutic applications as well as use in cosmetic industry. Different extraction solvents can dissolve different components depending on polarity. Here, acetone was used as solvent for extraction due to its low toxicity to bioassay studies, volatility and miscibility in water. It can extract out phenolics, tannins, saponins and flavonols which are known to have antimicrobial activities (Tiwari *et al*., 2011) [44]. Total phenolics and flavonoids content was estimated in the acetone extract and was found to be 8.2 mg gallic acid equivalent per gram dry weight and 4 mg quecertin equivalent per gram dry weight. The antifungal activity exhibited by acetone extract of *G. asitica* may be due to high content of phenols and flavonoids. Phenols exhibit antimicrobial activity by different mechanisms like substrate deprivation, binding to adhesins, complex with cell wall, enzyme inhibition, metal ion complexation or membrane disruption. Flavonoids also exhibit antimicrobial activity by forming complex with cell wall or by binding to adhesins.

Antioxidant plays a key role in providing protection to human beings against infections and degenerative diseases through their property of scavenging free radicals. The antioxidant activity of GALAE was determined by DPPH method. At room temperature DPPH is stable free radical with characteristic purple colour and maximum absorbance at 517 nm. The nitrogen free radical in DPPH is quenched by antioxidant to yield yellow coloured complex (1, 1-diphenyl-2-picrylhydrazine). The decolourization of purple colour is stoichiometric depending on the number of electrons gained. GALAE was found to contain 63.2 µg of antioxidant per gram of dry weight. Compared to ascorbic acid as standard, the antioxidant potential of GALAE was found to have 34.56 ascorbic acid equivalent antioxidant concentrations (AEAC). Sharma, N. and Patni, V. (2013) [39] reported DPPH radical scavenging activity of *G. asiatica* (acetone:water, 80:20 v/v) extract as 0.78±0.09mg mL^{-1}. Determination of inhibitory concentration i.e. the concentration of antioxidant needed to inhibit DPPH free radical formation is yet another method of ascertaining antioxidant ability. Thus IC_{50} of GALAE was also analyzed and found to be 127.5 µg/mL. The antioxidant potential of *Grewia asiatica* have been widely studied and reported from time to time by different researchers (Gupta *et al*., 2008[15]; Sharma, N. and Patni, V., 2013 [35]; Siddiqi *et al*., 2013) [37]. In 2011, Siddiqi *et al*. [36] reported the DPPH free radical scavenging activity of flavonoid fraction of *G. asitatica* to be 85 % at 20ppm which is comparable to our finding where percent reduction of DPPH free radicals is 70.7 % for the acetone extract of leaves. Free radicals such as superoxide anion, hydroxyl radicals, and hydrogen peroxide are reactive oxygen species, which is responsible for the initiation of oxidative stress and resulted in lipid peroxidation, through a process that is supposed to be implicated in the etiology of stress diseases such as diabetes, stroke, cancer, rheumatoid and arthritis heart diseases (Khan *et al*., 2009 [22]; Kumar S.S. and Nagarajan N., 2012 [25]; Parekh J. and Chanda

S., 2007 [31]; Tiwari S., 2008) [45]. Free radicals cause major damage to a wide range of other biomolecules including DNA which was found to be prevented to a great extent in the presence of GALAE. The efficacy was studied on salmon sperm DNA. In presence of Fenton's reagent, DNA was extensively damaged while in presence of plant extract along with Fenton's reagent, the extent of DNA damage was reduced and the protective effect was observed which are shown in figure 1 (lane 1-3). Similar findings were reported by Sharma K.V. and Sisodia R. (2010) [34], hence confirming our results that the acetone extract act as active scavenger of hydroxyl radicals and protects DNA against damage caused due to free radicals or radiation. The radioprotective and neuroprotective properties of *G. asaitica* have also been reported by Sisodia *et al*., (2008) [41]. Such health promoting attributes of *G. asaitica* be attributed to the presence of phenolics, flavonoids alkaloids, tannins, terpenoid and saponins. Hence it can be further explored for its possible application for treatment of diabetes, stroke, rheumatoid arthritis, cardiovascular diseases, cancer, reduce ageing effect etc.

Conclusion

The results of present study indicated that acetone extract of *G. asiatica* is having strong antifungal and antioxidant activity, which can be attributed to the presence of phenolics, flavonoids, alkaloids, tannins, terpenoids and saponins. Hence it can be further explored for its possible application for treatment of fungal diseases like dermatophytosis, aspergillosis, candidiasis, and zygomycosis, as well as metabolic disorders where reactive oxygen species have major contributions in pathogenesis of diseases like diabetes, stroke, rheumatoid arthritis, cardiovascular diseases, cancer and reduce ageing effect.

Conflict of interest: None

Author contribution

Swati Goswami has contributed in collection of all samples and raw plant materials, execution of experiments, data collection, data analysis and drafting of this research article. Dr. Reena Jain has major contribution in study design as well as providing chemicals and lab infrastructure. Dr. Harison Masih, is the principal investigator and major guide for Ph.D. of Miss. Swati Goswami. He has contributed in study design and drafting of this research article.

Table 1: Identification of fungal isolates and antifungal activity of GALAE as determined by agar well diffusion method, MIC and MFC.

Fungal cultures	Identification	Zone of inhibition(mm)	MIC (mg/mL)	MFC (mg/mL)
SH 2	*Aspergillus fumigates*	32±0.58	10	35
A 10	*Aspergillus niger*	25±1.53	70	140
SH 8	*Candida glabrata*	28±0.58	280	560
CHK 5	*Trichophyton sp.*	30±0.87	10	35
BHK 1	*Geotrichum spp.*	25±1.15	70	140
KHK 3	*Microsporum gypsium*	25±0.58	10	35
MHK 2	*Penicillium sp.*	30±1.73	70	140
A 3	*Trichophyton sp.*	20±1.53	10	35
BHK 2	*Trichophyton sp.*	29±1.00	10	35
CHK 2	*Trichophyton sp.*	30±1.15	280	560

Data of zone of inhibition is represented as mean ± standard deviation.

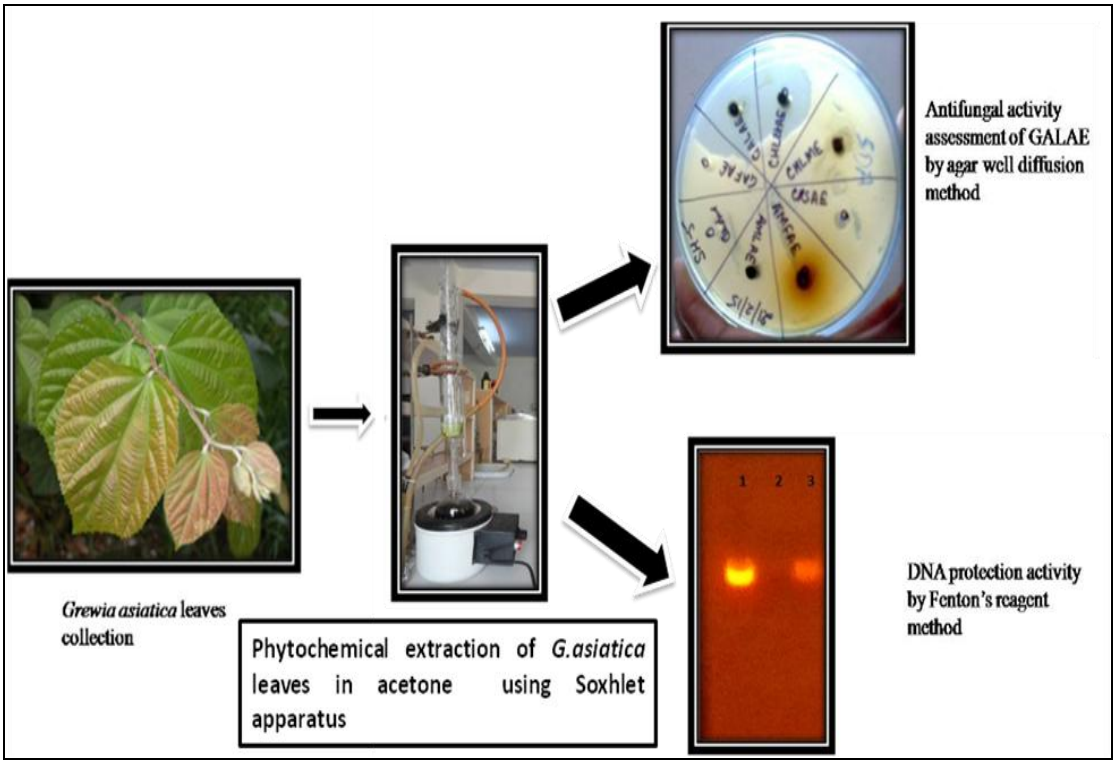

Fig 1: DNA protection active. Lane 1-Standard DNA without treatment, Lane 2-DNA+Fation's reagent, Lane 3-DNA+Fanton's reagent+ GALAE

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