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Full Length Research Paper

Nutritional and medicinal potential of *Grewia subinaequalis* DC. (syn. *G. asiatica*.) (Phalsa)

Jyoti Sinha*, Shalini Purwar, Satya Kumar Chuhan and Gyanendra Rai

Centre of Food Technology, University of Allahabad, India.

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***Grewia subinaequalis* DC. (syn. *G. asiatica*) (Phalsa) is a food plant and can also be used as an herbal medicine for the treatment of various diseases. In traditional folklore medicine, the fruit has been used as astringent, stomachic and cooling agent. When unripe, it has been reported to alleviate inflammation and was administered in respiratory, cardiac and blood disorders, as well as in fever. Root and bark has been prescribed for rheumatism and its infusion used as a demulcent. The leaves were applied on skin eruptions. A number of therapeutic research was carried out on different part of this plant like fruits, leaves, stem etc. The plant possess antioxidant, antidiabetic, antihyperglycaemic, radioprotective, antimicrobial, hepatoprotective, antifertility, antifungal, analgesic, antipyeretic and antiviral activities. This review focuses on the botanical description, phytochemistry, nutritional studies and pharmacological properties of this plant.**

Key words: *Grewia subinaequalis*, *Grewia asiatica*, phytochemistry, pharmacological properties.

INTRODUCTION

Fruits are regarded as a valuable food commodity with potential health benefits, being a rich source of carbohydrates, vitamins, antioxidants and minerals which are essential for an active and healthy life. Many fruits contain nonnutritive components such as flavonoids and other phenolic compounds that may provide protection against chronic diseases through multiple effects, which are as yet poorly understood (Tanaka et al., 1993). Fruits and vegetables exhibit their health-promoting properties by delaying the ageing process and by reducing the risk of various diseases including cardiovascular disorders, cancer, rheumatoid arthritis, lung diseases, cataract, Parkinson's or Alzheimer's disease (Szajdek and Borowska, 2008). Recent research has indicated that the people who eat higher amounts of fruits and vegetables have about one half the risk of cancer and less mortality from cancer (Steinmetz and Potter, 1991). Antioxidants present in different part of plants, are claimed to be helpful against cancer, cardiovascular and various chronic diseases. The presence of various biofunctional and chemo-preventive compounds in different parts of plant, believed to have health-boosting properties, are a major reason for their increased consumption. Fruits like ber, phalsa, apple and strawberry have been shown to possess antioxidant activity (Kaur and Kapoor, 2005). Medicinal plants have always been an exemplary source

*Corresponding author. E-mail: jyoti.sinha82@rediffmail.com

of drugs and many of the currently available drugs have been derived directly or indirectly from them. It is believed that the phytochemicals and vitamins largely responsible for their protective effects. Their activity is manifested by the scavenging ability of reactive oxygen species (ROS), such as hydroxyl, peroxide radicals, etc. (Rice-Evans et al., 1995).

Medicinal plants are particularly used by the traditional users since the ancient time but they do not have much scientific data. Hence considering the mentioned points, medicinal plant *Grewia subinaequalis* DC. (syn*. G. asiatica.*) (Phalsa) of genus *Grewia,* family Tiliaceae is selected for scientific review. The fruit, leave, bark of *Grewia* species have high medicinal values and are widely used for the treatment of various common diseases. *Grewia* is a genus of approximately 150 species of family Tiliaceae which include small trees and shrubs, distributed in subtropical and tropical regions of the world. The name *Grewia* was given due to Nehemiah Grew, one of the founders of plant physiology. Different species of *Grewia* are small tree which grow to 4 m or more in height and found in India, South Africa, Pakistan, Southeast Asia and USA etc. Most of the genuses of Tiliaceae family are wild and known for their fodder, fuel wood, craft works, timbers and therapeutic values viz. *Grewia flavescens* A. Juss, *Grewia villos* and *Grewia hirsuta. Grewia* is the only genus in family Tiliaceae with edible fruits. Extensively cultivated species for their fruit values are *G. subinaequalis* DC. (syn*. G. asiatica.*) and *Grewia tenax* (Frosk.) (Youngken, 1951). Medicinal values of *Grewia* species is due to the presence of different metabolites like saponins, coumarins and anthraquinone (Sharma and Patni, 2013).

From edible species of *Grewia*, *G. subinaequalis* DC. (syn*. G. asiatica.*) (Phalsa) are reputed to cure upset of stomachs, some skin and intestinal infections, cough, fever, diarrhoea, dysentery, jaundice, rheumatism and have mild antibiotic properties. The plant preparations are used for the treatment of bone fracture and for bone strengthening. Their root and fruits are well known household remedy for the treatment of osteoporosis, tissue and wound healing (Sharma and Patni, 2013). They have free radical scavenging activities which may be responsible for the therapeutic action against tissue damage (Kshirsagar and Upadhyay, 2009). Different classical texts found as medicinal plants of wound healing (Chopda and Mahajan, (2009). These plants are effective for the treatment of iron deficiency anaemia (Khemiss et al., 2006). Despite its diverse use, it has suffered notable disregard so here documenting the biological and chemical studies of one of the species of *Grewia* that is *Grewia subinaequalis* DC. (syn*. G. asiatica.*) (Phalsa) indigenous flora of India. Here we reviewed the phytochemistry, nutritional importance and therapeutic properties of this plant. This review will serve as a useful reference for further research on this important medicinal plant.

Botanical description and distribution

G. subinaequalis DC. (syn*. G. asiatica.*) is a 4 to 5 m tall shrub. The leaves are approximately 5 to 18 cm long and broad. The flowers are arranged in cymes of several together, the individual flowers are yellowish in color with five large (12 mm) sepals and five smaller (4 to 5 mm) petals. The flower has a diameter of about 2 cm (Sastri, 1956). Fruit is fleshy fibrous drupe, greyish purple at maturity, surface having black circular depressed spots with large stellate covering trichomes and rest of the surface with small stellate covering trichomes. Seeds are 1 or 2 in number, pointed at one end and grooved on the surface; seed coat stony hard. They are 1 or 2 chambered and endosperm is oily. Bark is greyish green, internally reddish brown, sometimes creamish in colour, thick, fibrous, tough and leathery. Leaf is shortly petioled, heart shaped, 5 to 7 nerved, main nerves connected by parallel venations, margin serrate, upper surface stellately pubescent, lower surface tomentose (Dey and Das, 1995).

Two distinct types, tall and dwarf, have been developed in India that differ with respect to various chemical and physical characteristics (Table 1). The juice yield is slightly higher in the tall type because it is directly related to edible portion, while more total sugars and non-reducing sugars were observed in the dwarf type. Tall type had more reducing sugars and titrable acidity and a greater amount of seed protein than the dwarf type (Dhawan, 1993).

Distribution in India

It is found throughout greater part of India, in salt range of Punjab, Western Himalaya up to 1000 m, N. Bengal, Bihar, Chota Nagpur, Orissa, Gujarat, Konkan, Deccan and South India.

Vernacular names in India

Hindi: Pharsa, Phalsa, Phulsa, Pharoah, Shukri, Dhamin, Parusha. Beng.- Phalsa, Shukri. Guj.- Phalsa. Kan.- Buttiyudippe, Tadasala. Ma1.- Chadicha. Mar.- Phalsa, Phalsi. Punj.- Phalsa. Tam.- Tadachi, Palisa. Te1.- Putiki,Phutiki, Jana, Nallajana. N.W.P.- Phalsa, harsiya. Oriya- Pharasakoli.Pers.- Falseh. Santhal- Jang olat. Sing.- Dowaniya. Sind.- Pharaho, Phalsa.Urdu- . Phalasah. (Bennet, 1987).

Nutritional composition

Fruits of *Grewia asiatica* (Phalsa) are low in calories and fat, and high in vitamins, minerals, and fiber. The detailed nutritional profile of fruit has been given in Table 2 (Yadav, 1999). Phytochemical screening revealed the

Table 1. Characteristics of tall and dwarf types of *Grewia asiatica* (Phalsa) (Dhawan 1993).

Content (%)	Tall	Dwarf
Edible portion	91.30	90.79
Seed	8.70	9.21
Juice yield	67.50	65.90
Pomace	32.50	34.10
Moisture	76.80	74.83
Total sugars	5.73	7.95
Reducing sugars	1.24	0.99
Non-reducing sugars	4.49	6.96
Titrable acidity	1.48	1.12
Fruit protein	3.13	1.89
Seed protein	8.75	7.00
Pulp protein	1.40	7.00

Source: Dhawan (1993).

Table 2. Nutritional values of *Grewia subinaequalis* DC. (syn. *G. asiatica.*) fruit. (Yadav, 1999).

Nutrients	Values/100 g
Protein (g)	1.57
Total lipid (fat) (g)	<0.1
Carbohydrate (g)	21.1
Ash (g)	1.1
Fibre (g)	5.53
Calcium (mg)	136
Iron (mg)	1.08
Phosphorus (mg)	24.2
Potassium (mg)	372
Sodium (mg)	17.3
Vitamin B1 (mg)	0.02
Vitamin B3 (mg)	0.825
Vitamin C (mg)	4.385
Vitamin B2 (mg)	0.264
Vitamin A (g)	16.11

Source: Yadav (1999).

presence of alkaloids, carbohydrates, glycosides, proteins and amino acids, saponins, steroids, acids, mucilage, fixed oils and fats. Fruits were observed for their characters under visible and ultralviolet light after treating with different chemical reagents and pharmacognostic parameters variable on the basis of their geographical origin were determined (Mukhtar, 2012). Nutritionally essential amino acids such as threonine and methionine are present in pulp and seeds, respectively, whereas phosphoserine, serine and taurine are the dominant amino acids in juice. The pulp contains higher concentrations of phosphoserine as compared to other free amino acids, while the hydrolyzed product contained aspartic acid, glycine, and tyrosine in large amount (Hasnain and Ali, 1988). Threonine was found in pulp but was missing in seed extract, whereas methionine was only present in seeds, indicating that the presence of methionine in fruit juice would be the result of adulteration. Phosphoserine, serine, and taurine were the dominant amino acids in juice (Hasnain and Ali, 1992). Chemical composition of seeds indicated that they contain bright yellow oil (5%). Fatty acid composition of this oil indicated the presence of palmitic (8%), stearic (11%), oleic (13.5%) and linoleic acids (64.5%) while small amount of unsaponifiable matter (3%) was also detected (Morton, 1987). *G. asiatica* fruits were analyzed for six micronutrients (Co, Cr, Cu, Ni, Zn and Fe) on fresh weight (FW) and dry weight (DW) (Table 3) (Khan, 2006). Iron was present in the highest concentration, while cobalt was present in the lowest amounts. Micronutrients play an important role in various physiological and metabolic processes of the human body.

Table 3. Mineral contents of *Grewia subinaequalis* DC. (syn. *G. asiatica.*) fruit. (Khan 2006).

Mineral	mg/100 g FW *	µg/100 g DW **
Cobalt	0.99	33
Chromium	1.08	36
Copper	0.48	16
Nickel	2.61	87
Zinc	144	48
Iron	140.8	1695

*FW = Fresh weight (Fresh fruit); **DW = Dry weight (After removal of moisture from fresh fruit). Source: Khan (2006).

Phytochemical activity

Fruits

Preliminary phytochemical screening of fruits indicated the presence of carbohydrate, tannins, phenolic compounds, flavonoids and vitamin-C in methanolic extract; flavonoids and fixed oil in petroleum ether extract; steroids in benzene extract; carbohydrate, tannins, flavonoids and phenolic compounds in ethyl acetate extract and carbohydrate, tannins, phenolic compounds and proteins in the aqueous extract (Gupta et al., 2006). Amino acids such as proline, glutaric acid, lysine and phenylalanine, and carbohydrates, like glucose, xylose, and arabinose were identified by paper chromatography in ethanol extract of fruit (Sharma et al., 2008).

Leaves

Phytochemical screening of the leaves revealed that their

Table 4. Total phenolic contents of various parts of fresh fruit (mg GAE/100 g). (Asghar et al., 2008).

Solvent used	Seed	Peel	Pulp
70% Acetone	1020	5080	2060
Water	991	3990	2050
60% Methanol	920	2852	1261
Dichloromethane	880	1980	1000
Ethyl acetate	2060	1094	895

Source: Asghar et al. (2008)

Table 5. Radical scavenging activities of the fractions derived from *G. asiatica*, by DPPH method. (Evans et al., 1995)

Fruit Sample	Fractions	Concentration of fractions			
		5 ppm	10 ppm	15 ppm	20 ppm
G. asiatica (Phalsa)	Fraction Ia	24±0.23	38±1.60	48±0.8	62±0.67
	Fraction II	30±0.89	50±0.98	58±0.67	70±0.98
	Fraction Ic	39±1.20	58±1.30	62±1.45	78±1.23
	Fraction Ib	48±2.30	62±1.56	75±1.89	85±3.23

Non anthocyanin fraction into flavanols (Fraction Ia), Anthocyanins (Fraction II), phenolic acids (Fraction Ic), and flavonols (Fraction Ib). Means ± SEM of triplicate assays. For a given fruit sample, values in the same column and row were significantly different at $p < 0.05$. Source: Evans et al. (1995).

petroleum ether extract contains diterpenes, glycosides and fats; chloroform extract contains alkaloids and glycosides, while ethanolic extract contains triterpenoids, sterols, flavonoids, saponins and tannins (Patil et al., 2011). Pharmacognostic evaluation of leaves reported total 5% of ash, consisting of water-soluble ash (2.5%) and acid-insoluble ash (2.1%) (Gupta et al., 2008). Phytochemical activity of bark and root are not yet determined.

Secondary metabolites and other compounds found in different parts of *Grewia asiatica* (Phalsa)

Fruits

Fruits of *G. asiatica* (Phalsa) contain pelargonidin 3,5-diglucoside, naringenin-7-*O*-β-D-glucoside, quercetin, quercetin 3-*O*-β-D-glucoside, tannins, catechins, and cyanidin-3-glucoside (Chattopadhyay and Pakrashi, 1975)

Flowers

β-sitosterol, quercetin, quercetin 3-*O*-β- D-glucoside, naringenin, naringenin 7-*O*-β-D-glucoside and a δ-lactone 3,21,24-trimethyl-5,7-dihydroxyhentriacontanoic acid were isolated from flowers (Lakshmi et al., 1976)

Leaves

Quercetin, kaempferol and a mixture of their glycosides were isolated from leaf extracts (Ali et al., 1982).

Stem and bark

The stem and bark contain betulin, lupeol, lupenone, and friedelin. β-amyrin and β-sitosterol were isolated from heartwood of *G. asiatica* (Phalsa) (Abou Zeid and Sleem, 2005).

Pharmacological activities

Antioxidant activity

An antioxidant is known to delay or prevent oxidation of substrate (Halliwell, 1990).

Fruits

Grewia asiatica (Phalsa) has a high content of antioxidants in fresh fruit like vitamin C, total phenolics, flavonoids, tannins and anthocyanins (Table 4) (Asghar et al., 2008). Radical scavenging activities of the different fractions [Non anthocyanin fraction into flavanols (Fraction Ia), Anthocyanins (Fraction II), phenolic acids (Fraction Ic), and flavonols (Fraction Ib)] derived from fruit of *G. asiatica* (Phalsa) by DPPH method given in Table 5

Table 6. Anthocyanin, flavonoid and total phenolic contents (data expressed as milligrams per 100 g of weight). (Siddiqi et al., 2011).

Fruit Sample	Fractions	Total phenolics[a]	Flavonoids[b]	Anthocyanins[c]
G. asiatica (Phalsa)	Fraction Ia	67±0.98	----	----
	Fraction II	288±0.07	178±0.23	----
	Fraction Ic	222±1.90	165±0.23	----
	Fraction Ib	151±0.40	100±0.90	72±6.0

(-) estimation not performed, Means ± SEM of triplicate assays. Values in the same column were significantly different at $p<0.05$, [a] Concentration based upon gallic as standard. [b] Concentration based upon catechin as standard. [c] Concentration based upon cyanidin-3-glucoside as standard. Source: Siddiqi et al. (2011).

Table 7. Antioxidant activity *G. asiatica* (fuit), fractions using β-carotene-linoleic acid assay. (Siddiqi et al., 2013).

Fruit Sample	Fractions	Total phenolics [a]
G. asiatica (Phalsa)	Fraction Ia	58±1.23
	Fraction II	65±2.30
	Fraction Ic	75±1.87
	Fraction Ib	89±2.00

Values are represented as mean ± standard error. Values in the same column and row were significantly different at $p < 0.05$. Source: Siddiqi et al. (2013).

(Siddiqi et al., 2013). All the fractions showed potent radical-scavenging activity. In all fractions the activity increased significantly with concentration ($p < 0.05$). The radical scavenging activity was maximum- 62-85% at 20 ppm ($p < 0.05$). The order of antioxidant activity of the different fractions were- Fraction Ib > Fraction Ic > Fraction II > Fraction Ia. The role of polyphenols as radical scavengers and in increasing the resistance of LDL oxidation involved in heart diseases have been demonstrated by many in vitro studies (Evans et al., 1995)

Antioxidant activity in the fruit of *G. asiatica* (Phalsa) can be explained on the basis of total phenolic contents, flavonoids and anthocyanins (Table 6) (Siddiqi et al., 2011). The order of antioxidant activity of the different fractions- Fraction Ib > Fraction Ic > Fraction II > Fraction Ia. Total phenolics were least in the Fraction Ia. Anthocyanins of about 72±6.0 mg/100gm was detected in Fraction Ib. Polyphenolics in foods are more efficient antioxidants than vitamins C & E, and β-carotene (Vinson et al., 1995). The order of flavonoids were - Fraction II > Fraction Ic > Fraction Ib. Flavonoids, a family of polyphenolic compounds, are widely distributed pigments, possessing anti-radical and chelating properties. They can scavenge free hydroxyl and peroxy radicals or may extract iron ions to depress superoxide-driven Fenton reaction (Afanasev et al., 1989). It is established that antioxidant potential of lots of fruits is based on their flavonoid contents (Wang, 1996). All fractions of *G. asiatica (*fruit) effectively shows the oxidation of β-carotene in the linolenic emulsion system ($p < 0.05$) (Table 7) (Siddiqi et al., 2013). β-carotene oxidation was shown by *G. asiatica* fractions from 58 to 89%, highest in Fraction Ib and lowest in Fraction Ia. An antioxidant is known to delay or prevent oxidation of substrate. The antioxidant activity is dependent upon the reducing ability (Tanaka et al., 1998). Table 8 shows the reducing power of the fractions derived from *G. asiatica* (Phalsa) fruits using potassium ferricyanide reduction method. The absorbance of fractions were highest at 50 ppm concentration at 700 nm ranged from 1.53 to 3.1. The reducing property relates to the presence of reductones (Pin-Der, 1998). It not only break the free radical sequence by providing a single hydrogen but also quench peroxide formation by reacting with precursors of peroxide (Gordon, 1990).

The antioxidant activity of a methanol extract of the fruit of *G. asiatica* was evaluated by various assays indicated that fruit possesses considerable antioxidant activities. Higher amounts of total flavonoid content (4.608 QE mg/g), total phenolic content (144.11 mg GAE/g) and total antocyanin contents (4.882 mg/kg) were observed, while the antiradical activity against DPPH (84.83%) and peroxide radical (37%) was observed. Values noted for TEAC (269.038 mMTE) and FRAP (4.14 GAE/g) were also comparatively greater than that of various other plant species (Srivastava et al., 2012)

Fruit pomace

The fruit pomace was assessed for total flavonoids,

Table 8. Reducing power of the fractions derived from *G. asiatica* (Phalsa) fruits. (Pin-Der, 1998).

Fruit Sample	Fractions	Concentration of fractions			
		5 ppm	10 ppm	25 ppm	50 ppm
G. asiatica (Phalsa)	Fraction Ia	0.52±0.09	0.75±0.05	1.30±0.10	1.53±0.06
	Fraction II	0.80±0.02	1.00±0.04	1.60±0.10	2.00±0.12
	Fraction Ic	0.85±0.09	1.40±0.13	2.00±0.05	2.60±0.10
	Fraction Ib	1.00±0.05	1.80±0.06	2.40±0.14	3.10±0.05

Values are represented as mean ± standard error. Values in the same column and row were significantly different at $p < 0.05$ except where the same superscripts have been used to show no significant difference. Source: Pin-Der (1998).

Table 9. Antioxidant Activity of *Grewia asiatica* (Phalsa) Leaves Extracts Using DPPH Method. (Gupta et al., 2007)

Test compounds	IC_{50} values ± SE* (µg/ml)
Petroleum ether extract	249.60±7.37
Benzene extract	16.19±2.13
Ethyl acetate extract	26.17±1.49
Methanol extract	27.38±1.80
50 % Methanol crude extract	56.40±3.98
Aqueous crude extract	176.14±5.53
Ascorbic acid	78.17±4.05
Quercetin	53.60±1.79

*Average of 10 determinations. Source: Gupta et al. (2007).

Table 10. Antioxidant activity Of *Grewia asiatica* (Phalsa) Leaves Extracts Using Nitric Oxide Radical Inhibition Assay. (Gupta et al., 2007)

Test compounds	IC_{50} values ± SE* (µg/ml)
Petroleum ether extract	22.12±2.65
Benzene extract	27.00±1.62
Ethyl acetate extract	47.38±5.88
Methanol extract	56.85±6.16
50 % Methanol crude extract	72.75±13.76
Aqueous crude extract	152.75±5.76
Ascorbic acid	20.50±1.16
Quercetin	19.50±1.85

*Average of 10 determinations. Source: Gupta et al. (2007).

alkaloids, saponins and tannins and the values observed per dry matter were 12.42 ± 0.56 (CE mg/g), 1.56 ± 1.2 (g/100 g), 1.05 ± 0.96 (g/100 g) and 0.52 ± 1.25 (g/100), respectively (Gupta et al., 2013). These results indicate that even material considered as a waste has substantial amount of antioxidants. An aqueous extract of fruit exhibited total phenol content (CE) and total flavonoid content (GAE) of 5.25 and 0.13, respectively. The results indicated comparatively higher contents as compared to other 21 extracts analyzed simultaneously (Das, 2012).

Leaves

The antioxidant activity of the leave extract and the standards were assessed on the basis of the radical scavenging by using DPPH method and nitric oxide radical inhibition assay free radical (Bang et al., 2001). The successive leaves extracts of *G. asiatica* (Phalsa) exhibited antioxidant activity in the DPPH and the nitric oxide radical inhibition assay as evidenced by the low IC_{50} values (Tables 9 and 10) (Gupta et al., 2007). The

successive extracts such as petroleum ether, benzene, ethyl acetate, methanol, water and 50 % crude methanol extracts exhibited IC50 values of 249.60 ± 7.37, 16.19 ± 2.132, 26.17 ± 1.49, 27.38 ± 1.80, 176.14 ± 5.53 and 56.40 ± 3.98 µg/ml, respectively in DPPH and 22.12 ± 02.65, 27.00 ± 01.62, 47.38 ± 05.88, 56.85 ± 06.16, 152.75 ± 5.76 and 72.75 ± 13.76 µg/ml, respectively in nitric oxide radical inhibition assays. These values were more than those obtained for ascorbic acid and quercetin, used as standards.

Free radical oxidative stress has been implicated in the pathogenesis of a wide variety of clinical disorders, resulting usually from deficient natural antioxidant defenses (Halliwell and Gutteride, 1989). Among the 6 extracts *G. asiatica* (Phalsa) leaves and 2 standards tested for antioxidant activity using DPPH method, the benzene and ethyl acetate successive extracts showed the maximum antioxidant activity with IC_{50} values of 16.19 ± 2.13 and 26.17 ± 1.49 µg/ml, respectively. The methanol extract also showed antioxidant activity with IC_{50} values 27.38 ± 1.80 µg/ml. The 50% methanol and distilled water crude extracts showed IC_{50} values of 56.40 ± 3.98 and 176.14 ± 5.53 µg/ml, respectively. However, petroleum ether extract showed lowest anti oxidant activity with an IC_{50} value of 249.60 ± 7.37 µg/ml. The known antioxidants ascorbic acid and quercetin exhibited IC_{50} values of 78.17 ± 4.05 and 53.60 ± 1.79 µg/ml, respectively. Thus antioxidant capacity of *G. asiatica* (Phalsa) is due to presence of total phenolic contents, flavonoid contents, tannin contents and the anthocyanin contents which depends on several factors such as different genotype, growing condition, agronomic practices employed, season, maturity, post-harvest storage and processing conditions and solvent used for extraction.

Antimicrobial activity

Fruit and bark

An ethanol extract of *G. asiatica* bark and fruit posses antimicrobial potential against *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pneumoniae* and six Gram negative strains, *Escherichia coli*, *Proteus vulgaris*, *Proteus mirabilis*, *Salmonella typhi para A*, *Salmonella typhi para B* and *Shigella dysenteriae*, resulting active against *S. aureus*, *E. coli* and *P. vulgaris* (Israr et al., 2012).

Pulp and peel

Polyphenolics were isolated from crude methanol extracts of *G. asiatica* pulp and peel and further fractionated into ethyl acetate fraction. This was further divided into three groups: neutral fraction A, comprising flavanols and other polyphenolics, neutral fraction B comprising flavonols, acidic phenolics fraction and anthocyanin fraction. These major fractions were analyzed for their antimicrobial effects. All fractions showed significant antibacterial activity, except the fraction containing anthocyanins. The most susceptible strain was *Staphylococcus aureus* amongst the Gram-positive, while amongst the Gram-negative bacterial strains, the most susceptible was *Salmonella typhi*. The most resistant Gram-positive bacteria was Bacillus subtilis, while most resistant gram-negative strain was *E. coli*; both *Aspergillus* strains were substantially inhibited by all fractions. Fraction containing flavanols and other polyphenos was evaluated for its antifungal potential. No growth of *Trichophyton mentagrophytes* and *Trichophyton rubrum* was observed. Inhibition of *Aspergillus* strains by the fractions supports that the chemicals present in the fractions could be effective in the prevention of aflatoxins production in food products. Being the most active, phenolic acid fraction was also tested for its antifungal activity against six fungal pathogens, namely *Penicillium notatum*, *Aspergillus niger*, *A. flavus, Microsporum gypseum, T. mentagrophytes* and *T. rubrum.* The fraction substantially inhibited all the tested fungal species (Siddiqi et al., 2011).

Pomace

Different extracts of *G. asiatica* pomace were assayed against Gram positive (*Bacillus subtilis*, *B. cereus*, *Staphylococcus aureus*, *Enterococcus faecalis*) and Gram negative bacteria (*Escherichia coli*, *Listeria monocytogeneses*, *Salmonella typhimurium*, *Shigella flexneri* and *Pseudomonas aerugenosa*). Gram positive were more susceptible than Gram negative bacteria (Gupta et al., 2012). Gram-positive bacteria are usually more sensitive to crude extracts and bioactive constituents because of the specific structure of their cell walls.

Leaves

Ethanolic extract of leaves showed antibacterial and antifungal activities. The extract showed potent results against strains; *Proteus mirabilis*, *Citrobacter* sp., *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi*, *Micrococcus luteus*, *Staphylococcus aureus*, and *Bacillus subtilis*. The extract showed significant activity against nine fungal strains; *Aspergillus effusus*, *A. parasiticus*, *A. niger*, *Saccharomyces cerevisiae*, *Candida albicans*, *Yersinia aldovae*, *Fusarium solani*, *Macrophomina phaseolina*, and *Trichophyton rubrum* (Zia-Ul-Haq et al., 2011). *G. asiatica* leaves possess antimicrobial potential and are therefore used to treat skin rashes and pustular eruptions (Zia-Ul-Haq et al., 2012).

Table 11. Effect of methanol extract of Grewia asacita (MEGA) on Body weight, Mean survival time, ILS, Tumor volume, Packed cell volume, Viable and onviable tumor cell count in EAC Bearing mice. (Nair and Panikkar, 1990).

Treatment	Dose (mg/kg)	Total body weight (g)	Mean survival time (days)	% Increase of life Span (ILS)	Tumor volume (ml)	Packed cell volume (ml)	Viable cell (×104 cells/ml)	Non Viable cell (×104)
EAC control (0.9% Nacl)	5 ml	22.12 ± 0.21	18.51 ± 0.38	-	3.29 ± 0.01	2.83 ± 0.04	18.11 ± 0.02	10.9 ± 0.10
MEGA (250 mg/kg) + EAC	250	20.03 ± 0.84	26.14 ± 0.41	41.22	2.50 ± 0.51	1.70 ± 0.51*	11.80 ± 0.07*	14.1 ± 0.60*
MEGA (500 mg/kg) + EAC	500	19.60 ± 0.94	29.83 ± 0.69	61.16	1.14 ± 0.23	1.20 ± 0.12*	9.82 ± 0.51*	16.5 ± 0.60*
Cisplatin + EAC	5	20.54 ± 1.24	35.78 ± 0.56	93.30	0.60 ± 0.20	0.20± 0.01*	1.25 ± 0.10*	21.56 ± .20*

EAC=2× 106 cells/mouse , *P<0.05 for the treated groups when compared with EAC, Values are mean±SEM, n=5. Source: Nair and Panikkar (1990).

Anticancer activity

Fruits

Aqueous extracts of fruits showed significant anticancer
activity against liver cancer and breast cancer. The *in vitro* cytotoxic activity was determined by methylthiazolyl tetrazolium (MTT) assay using epidermal kidney (HEK-293), breast (MCF-7), cervical (HELA), lung (NCI-H522) and laryngeal (Hep-2) cancer cell lines. The fruit extract was found to be active on lung (IC_{50} = 59.03 µg/ml) and breast (IC_{50} = 58.65 µg/ml) cancer cell lines. The results suggest that the fruits extract as a potential agent for the management of human cancer (Marya et al., 2011).

Leaves

The *in vitro* antitumoral and cytotoxic activities of a methanol extract of *G. asiatica* leaves has been assessed by MTT assay against four human cancer cell lines: acute myeloblastic leukemia (HL-60), chronic myelogenic leukemia (K-562), breast adenocarcinoma (MCF-7) and cervical epithelial carcinoma (Hela), with IC_{50} values of 53.70, 54.90, 199.5 and 177.8, 89.12, respectively. The intraperitoneal administration of 250 and 500 mg/kg of extract to male Swiss albino mice increased the life span of Ehrlich's ascites carcinoma (EAC) tumor bearing mice by 41.22% and 61.06%, respectively. The same extract was found to be active in preventing the EAC development in mice in a dose dependent manner (Kakoti et al., 2011). Aqueous extracts of leaves showed significant anticancer activity against liver cancer and breast cancer. Leaf extract was active against breast (IC50 =50.37 µg/mL) and Hep-2 (IC50 = 61.23 µg/mL) cancer cell lines. The results suggest that leaf extract as a potential agent for the management of human cancer (Gupta et al., 2013).

Pomace

The *in vitro* cytotoxic activity (IC_{50}) of pomace methanol extract evaluated against cervical epithelial carcinoma (HeLa), breast adenocarcinoma (MCF-7) and hepatocellular carcinoma cells (HepG-2) was > 100, 68.91 and > 250 µg/ml, respectively. These results suggested that *G. asiatica* pomace possessed promising anticancer activity that substantiated its ethno-medicinal use and may provide new molecules for treatment of these cancers (Gupta et al., 2013).

Antitumor activity

Leaves

Antitumor activity of the methanolic extracts of *Grewia asiaica* (Phalsa) (MEGA) leaves was determined using ascites tumor model. (Table 11) (Nair and Panikkar, 1990). Animals were divided into four groups of five animals in each group. All the animals were injected intraperitoneally (i.p.) with 2 × 106 cells/ml viable EAC cells in phosphate buffer saline (aspirated from 15 days old EAC ascites tumor in mice). After 24 hrs of tumor inoculation, MEGA at a dose of 250 and 500mg/kg body weight was administered orally and this was continued for 10 consecutive days. The group administered with vehicle alone (0.9% w/v Nacl) was maintained as control. Cisplatin (2mg/kg b.w.) i.p was used as standard reference drug. The blood parameters and the ILS (increase in life span), tumor volume, tumor cells count, viable and non-viable cells, mean survival time of the control and tumor groups were noted and compared to that of that of standard Cisplatin. The ILS was determined using the formula % ILS = (1 –T/C) × 100 where T is the mean survival time of treated group and C that of control group (Nair and Panikkar, 1990).

The result showed that leave extracts showed

Table 12. Effect of MEGA on various cancer cell lines. (Bibhuti et al., 2011)

Cancer Cell Lines	MTT Assay MEGA (IC_{50}, g/ml)	Trypan blue exclusion assay MEGA (IC_{50}, g/ml)
HL – 60	53.70	89.12
K – 562	54.90	51.11
MCF – 7	199.5	85.11
Hela	177.8	128.8

Source: Bibhuti et al. (2011).

cytotoxicity towards tumor cells and antitumor activity. Prolongation of the life span with the MEGA treatment is a clear suggestive of the anticancer activity of the plant. Body weight of the tumor bearing mice was also found to be decreased with the MEGA administration. The doses of 250 mg/kg b.w and 500mg/kg b.w, p.o. were selected based on the preliminary studies carried out. Antitumor activity of this plant may be may be either though induction of apoptosis or by inhibition of neovascularisation. The presence of various phytoconstituents in the plant may attribute the observed anticancer activity. Further investigation is being carried out for finding the phytochemical entities responsible for eliciting the effects (Bibhuti et al., 2011). Research showed that antitumor activity was determined only on *G. asiaica* (Phalsa) leaves.

Cytotoxicity activity

Leaves

Cytotoxicity is the quality of being toxic to cells. Leave extract of *G. asiaica* (Phalsa) (MEGA) showed significant cytotoxicity effect against the tested human cancer cell lines as represented in Table 12 (Bibhuti et al., 2011). The IC_{50} value of the MEGA by MTT was calculated by regression analysis and was found to be 53.70 µg/ml in HL-60, 54.9 µg/ml in K-562, 199.5 µg/ml in MCF-7 and 177.8 µg/ml in Hela cells, respectively. The IC_{50} value of MEGA by and trypan blue exclusion assay was calculated by regression analysis and found to be 89.12 µg/ml in HL-60, 51.11 µg/ml in K-562, 85.11 µg/ml in MCF-7 and 128.8 µg/ml in Hela cells, respectively. In the EAC studies, the MEGA treated group showed decrease in the viable cell count as compared with the EAC treated group. Result showed that leave extracts showed cytotoxicity towards tumor cells. The MEGA was found to be active against all the four human cells lines as observed from the cytotoxicity assays. Research showed that cytotoxicity activity determined only on *G. asiaica* (Phalsa) leaves.

Radioprotective activity

The increasing use of nuclear radiation for human welfare despite its beneficial effects, has some undesirable side effects so there is need to check the side effects for the same. Search for the chemical agents that are able to protect human beings from the ionizing radiation is a key issue in radiation biology (Nair et al., 2001).

Fruit

The effects of methanolic extract of *G. asiaica* (Phalsa) fruit were evaluated in brains of Swiss albino mice for their radioprotective effects. The mice was divided in different groups: group I received no treatment, group II was orally supplemented, once daily, of the dose of 700 mg/kg for fifteen consecutive days; group III (control) received distilled water orally equivalent to the extract for fifteen days, then was exposed to 5 Gy of gamma radiation, and group IV, to which the extract was administered orally for 15 consecutive days, once daily, and exposed to single dose of 5 Gy of gamma radiation. Mice were sacrificed at different post irradiation intervals (1, 3, 7, 15 and 30 days). Brains were removed for the estimation of glutathione (GSH) and lipid peroxidation (LPO). Extract supplementation controlled the increase of LPO due to radiation, approximately by 5% at day 30 post irradiation, whereas radiation induced depleted levels of GSH could be raised by 14.57% 30 days after, thereby indicating that the extract may control the radiation induced disturbances (Ahaskar and Sharma, 2006).

The radioprotective efficacy of the same extract against whole body gamma radiation was studied in Swiss albino mice. After drug toxicity testing, the oral administration of 700 mg/kg/day of extract for 15 consecutive days before exposure to 10 Gy of γ- radiation provided maximum protection, as evidenced by the highest number of survivors 30 days post irradiation. $LD_{50/30}$ value of 6.21 for irradiation alone (control) and 9.53 for *G. asiatica* (Phalsa) extract + irradiation group (experimental) was obtained, while the dose reduction factor calculated was 1.53. The mice of experimental group exhibited significant modulation of radiation-induced decreases of reduced glutathione (GSH) and radiation-induced increase in lipid peroxidation (LPO) in the whole brain and liver at 24 h after radiation exposure (Ahaskar et al., 2007). Radioprotective efficacy of a fruit extract was

studied against radiation induced biochemical alterations in mice cerebrum. Mice were sacrificed at different intervals (1, 3, 7, 15 and 30 days) and cerebrum was tested for the estimation of glutathione (GSH), lipid peroxidation (LPO) and proteins. The extract showed a protection against the biochemical changes in mice cerebrum. Radiation induced increase in the levels of LPO was significantly reduced by extract post-treatment. Similarly, radiation-induced depletion in proteins was significantly controlled by extract administration (Ahaskar et al., 2007).

The radioprotective effects of a fruit extract were studied in Swiss albino mice divided into five groups: control (I); extract treated (700 mg/kg for 15 days) (II); irradiated (5 Gy) (III); extract + irradiated (IV) and irradiated extract treated (V). The irradiation of animals resulted in a significant elevation of lipid peroxidation in terms of thiobarbituric acid reactive substances (TBARS) content and depletion in glutathione (GSH) and protein levels, as compared to control group. The treatment of mice with extract before and after irradiation caused a significant depletion in TBARS content, followed by a significant elevation in GSH and protein concentration in the intestine and testis of mice at all post-irradiation autopsy intervals, in comparison to irradiated mice. Significant protection of DNA and RNA in testis was also noticed. The extract was found to have strong radical scavenging activity in DPPH and O^{2-} assays and also showed *in vitro* radioprotective activity in protein carbonyl assay, in a dose-dependent manner (Ahaskar et al., 2007).

The radioprotective effect of the fruit extract was studied in mice testis by histopathological examination. Irradiation of animals led to significant decrease in testis weight, whereas the treated group showed significantly higher values in comparison to the irradiated group. The histopathological study showed that the group irradiated showed significantly lower spermatogonia “A”, spermatogonia “B”, spermatocytes and spermatid count. These counts were higher in extract preand post treated irradiated group, in comparison to the respective irradiated group, till last autopsy interval (30 days post irradiation). This can indicate that the extract has protective potential to the damaging effect of radiation to the testis (Sharma and Sisodia, 2010).

Fruit pulp

The radioprotective effect of a fruit pulp extract of *G. asiatica* in mice blood against radiation induced hematological and biochemical alterations was evaluated. Mice were divided into four groups, group I (normal) without any treatment, group II orally supplemented with extract once daily at the dose of 700 mg/kg for 15 days, group III (control) only irradiated and group IV (drug+IR) to which the extract was administered as to group II and then exposed to 5 Gy of gamma radiation. Mice were sacrificed at 24 and 72 h post irradiation. Radiation induced deficit in different blood constituents GSH, GSH-Px, sugar, and protein levels in serum were significantly increased, whereas radiation induced elevation of lipid peroxidation and cholesterol level was markedly decreased in extract pretreated animals than control group. The extract provided protection against radiation-induced alterations in blood of Swiss albino mice (Singh et al., 2007).

In another study, the radioprotective effects of a fruit pulp extract on cerebrum of Swiss albino mice exposed to 5 Gy gamma radiation were investigated. Cerebra of mice was observed for various parameters after sacrificing in interval times of 1 to 30 days. Radiation-induced increase in the levels of lipid peroxidation of mice cerebrum was significantly reduced by extract pretreatment. The radioprotective effects of the same extract were investigated in mice blood and liver, by the evaluation of glutathione (GSH) and lipid peroxidation. The results indicated that extract post-treatment protects liver and blood against radiation-induced damage, by inhibiting glutathione depletion and decreasing lipid peroxidation levels that attended normal levels by day 30 post-treatment. The magnitude of recovery from oxidative damage in terms of TBARS and GSH content was significantly higher ($p < 0.001$) in the irradiated + extract-treated group (Sisodia et al., 2008). The protection afforded with *G. asiaica* (Phalsa) in the biochemical activity of liver and brain, the present study may prove to be beneficial for the clinical use of such dietary compounds as radioprotectors.

Hepatoprotective activity

Hepatoprotection or antihepatotoxicity is the ability to prevent damage to the liver. This damage is known as hepatotoxicity.

Fruit

The hepatoprotective effect of a fruit extract of *G. asiatica* was studied in mice testis. The irradiation resulted in a significant decrease in DNA and RNA levels in comparison to controls. Administration of extract before and after irradiation caused a significant elevation in liver DNA and RNA levels. Photomicrography of liver showed that pre- and post- administration of extract provided protection against radiation. The irradiation of animals resulted in a significant depletion in the DNA and RNA level at all intervals studied, 1-30 days in comparison to control group. Treatment of mice with *G. asiatica* fruit extract before and after irradiation caused a significant elevation in liver DNA and RNA level in comparison to irradiated mice. Photomicrograph of liver histology also

showed that pre and post supplementation of *G. asiatica* provides protection against radiation. Similarly counting of different type hepatocytes also showed that *G. asiatica* protect the liver against radiation (Sharma and Sisodia, 2010).

Antihyperglycemic and antidiabetic activity

Fresh fruits

Aqueous extracts of fresh fruits were studied for carbohydrate digesting enzymes (α-glucosidase and α-amylase) inhibitory properties. IC_{50} values (mg/ml) against α-amylase and α-glucosidase were 8.93 and 0.41, respectively, resulting in a moderate α-amylase and high α-glucosidase inhibitory activities as compared to other 21 extracts (Das and De, 2012).

Fruit pomace

Fruit pomace was extracted with aqueous acetone (80:20), aqueous methanol (80:20) and a solvent mixture (ethanol/hexane/water, 80:10:10). All these three extracts were tested for their potential antidiabetic activity by α-amylase inhibition assay. IC_{50} (mg/ml) values observed are 45.7, 85.2 and 138.1, respectively. Inhibition of α-amylase is believed as a strategy for diabetes and obesity management as it reduces blood sugar level In alloxan-induced hyperglycemic rabbits (Gupta et al., 2013).

Fruit, stem bark and leaves

Ethanolic extracts of fruit, stem bark and leaves of *G. asiatica* showed antihyperglycemic activity in alloxan-induced hyperglycemic rabbits. Oral dministration in suspension and capsule at 200 and 100 mg/kg, respectively of fruit, stem bark and leaves extract reduced serum glucose levels of rabbits. The results suggest that the fruit, stem bark and leaves of *G. asiatica* possess significant antihyperglycemic activity. Blood glucose, blood cholesterol and triglycerides levels were found to be significantly reduced by ground herbal drugs including *G. asiatica* (bark) in normal and alloxan induced diabetic rabbits (Parveen et al., 2012).

Leaves

G. asiatica leaves is used as a medicine for the treatment of diabetes mellitus. Glyburide is a potent, second-generation, oral sulfonylurea antidiabetic agent used as an adjunct to diet to lower blood glucose levels in patients with diabetes mellitus. The hypoglycaemic action of glyburide is due to stimulation of pancreatic islet cells, which results in an increase in insulin secretion. The effects of sulfonylurea are initiated by binding to and blocking on ATP sensitive K+ channel, which have been cloned. The drugs thus resemble physiological secretagogues (e.g. glucose, leucine) which also lower the conductance of this channel. Reduced K+ conductance causes membrane depolarization and influx of Ca^{+2} through voltage sensitive Ca^{+2} channel. Prolonged administration of glyburide also produces extrapancreatic effects that contribute to its hypoglycaemic activity (Shah et al., 2006).

The reduction in serum glucose from basal value (before) at 6 h after glyburide and EtGA (ethanolic extract of *G. asiatica*) (200 and 400 mg/kg) were 127.11, 172.63 and 213.54, respectively. The onset of the antihyperglycaemic effect of glyburide was at 2 h and EtGA (400 mg/kg) was at 4 h; the peak effect was 6 h but the effect waned at 24 h. EtGA (400 mg/kg) resulted in lowered serum glucose at 24 h (Table 13) (Bhangale et al., 2010).

In the subacute study, repeated administration (once a day for 28 days) of EtGA and glyburide caused significant reduction in the serum glucose level as compared to vehicle treated group. On the 21st day, EtGA (200 and 400 mg/kg) and glyburide showed significant reduction in the serum glucose level as compared to vehicle treated group. On the 35th day, the reductions in serum glucose level of glyburide and EtGA (100, 200 and 400 mg/dl) were 268.62, 94.16, 171.88 and 234.57, respectively (Table 14) (Bhangale et al., 2010). The body weight of vehicle treated diabetic animals decreased during the study period. Glyburide and EtGA (400 mg/kg) prevented the decreased in body weight of diabetic animals (Table 15) (Bhangale et al., 2010).

Subacute treatment for 35 days with the EtGA in the treated doses brought about improvement in body weights indicating its beneficial effect in preventing loss of body weight in diabetic animals (Xie et al., 2003). The ability of EtGA to prevent body weight loss seems to be due to its ability to reduced hyperglycaemia. In the oral glucose tolerance test, administration of glucose load (2.5 g/kg) increased serum glucose levels significantly after 30 min in non diabetic and alloxan treated diabetic mice. Glyburide (10 mg/kg) and EtGA (100, 200 and 400 mg/kg) produced a significant increase in the glucose threshold within 60 min, which was then reversed at 120 min after glucose loading nondiabetic (Table 16) as well as alloxan induced diabetic animals (Table 17) (Bhangale et al., 2010).

EtGA significantly enhanced glucose utilization in OGTT in both nondiabetic and diabetic animals. From the data obtained OGTT, it is clear that administration of EtGA effectively prevented the increase in serum glucose level without causing a hypoglycaemic state. The effect may be due to restoration of the delayed insulin response. The results of both acute and subacute study

Table 13. Effect of EtGA leaves on serum glucose level in alloxan-induced diabetic mice (acute study). (Bhangale et al., 2010).

Treatment (mg/kg)	Mean fasting glucose level (mg/dl) ± SEM				
	0 h	2 h	4 h	6 h	24 h
Vehicle	441.19±15.10	450.17±9.58	458.83±14.45	462.52±16.81	468.17±15.93
Glyburide (10)	441.67±5.25	360.18±17.62*	336.95±19.39***	228.54±21.01***	369.31±18.18**
EtGA (100)	482.44±15.05	476.72±14.46	450.16±19.70	434.32±20.71	464.12±25.85
EtGA (200)	477.85±17.82	450.52±13.63	417.72±18.16	350.74±18.48**	433.01±28.29
EtGA (400)	489.58±15.35	397.61±16.29	350.13±19.73**	316.95±29.98***	372.62±23.23*

n = 10, data was analyzed by two-way ANOVA with post hoc Dunnett's test using Graphpad Instat software, *P<0.05, **P<0.01, ***P<0.001 as compared with vehicle-treated animals the significance level was considered at 2a=0.05. Source: Bhangale et al. (2010).

Table 14. Effect of EtGA leaves on serum glucose level in alloxan-induced diabetic mice (Subacute study). (Bhangale et al., 2010).

Treatment (mg/kg)	Mean fasting glucose level (mg/dl)±SEM					
	0 day	7 day period	14 day	21 day	28 day	After day 7 rest period
Vehicle	441.19±15.10	483.52±19.30	501.74±17.16	514.50±16.45	529.14±13.07	528.86±12.21
Glyburide (10)	441.67±5.25	344.29±17.81***	292.16±27.87***	245.47±30.44***	192.69±22.24***	172.85±21.82***
EtGA (100)	482.44±15.05	448.31±20.21	430.29±24.53	454.97±20.97	398.40±19.33***	388.28±32.42***
EtGA (200)	477.85±17.82	421.96±16.44	392.72±17.53**	348.38±22.83***	330.64±15.69***	305.97±27.44***
EtGA (400)	489.58±15.35	389.56±17.58*	366.84±19.11***	330.08±19.95***	317.94±29.15***	255.01±27.72***

n = 10, data was analyzed by two-way ANOVA with post hoc Dunnett's test using Graphpad Instat software, *P<0.05, **P<0.01, ***P<0.001 as compared with vehicle-treated animals. The significance level was considered at 2a=0.05. Source: Bhangale et al. (2010).

Table 15. Effect of EtGA leaves on body weight in alloxan-induced diabetic mice. (Bhangale et al., 2010).

Treatment (mg/kg)	Mean body weight (g)±SEM					
	0	7	14	21	28	After day 7 rest period
Vehicle	30.50±0.43	27.50±0.62	27.00±0.26	26.00±0.45	22.00±1.06	18.00±0.82
Glyburide (10)	30.00±0.58	29.00±0.82	31.00±0.89**	30.00±0.68**	29.00±0.82***	30.00±1.37***
EtGA (100)	29.00±0.26	28.00±0.68	28.00±0.68	27.00±0.52	27.00±1.15	25.00±1.06
EtGA (200)	30.00±0.52	30.00±0.37	29.00±0.86	28.00±1.24	28.00±1.41***	26.00±0.77***
EtGA (400)	25.00±0.37	30.00±0.73	29.00±0.52	30.00±0.89**	30.00±0.73***	31.00±1.13***

n = 10, data was analyzed by two-way ANOVA with post hoc Dunnett's test using Graphpad Instat software, **P<0.01, ***P<0.001 as scompared with vehicle-treated animals. The significance level was considered at 2a=0.05. Source: Bhangale et al. (2010).

Table 16. Effect of EtGA leaves on oral glucose tolerance test (OGTT) in non diabetic mice. (Bhangale et al., 2010).

Treatment (mg/kg)	Mean fasting glucose level (mg/dl) ± SEM				
	Before glucose	0 min	30 min	60 min	120 min
Vehicle	129.31±10.31	334.68±14.66	261.31±9.98	219.01±7.91	158.71±10.40
Glyburide (10)	121.52±11.32	315.30±9.48	191.95±8.06***	165.64±9.98***	172.86±11.18
EtGA (100)	113.98±6.28	299.09±10.08	225.86±8.75	183.68±8.57	149.20±5.80
EtGA (200)	113.12±7.41	327.25±6.34	224.57±7.42*	156.80±4.56***	160.46±7.26
EtGA (400)	117.63±7.17	333.39±14.78	201.78±7.12***	142.11±5.70***	154.73±7.71

n = 10, data was analyzed by two-way ANOVA with post hoc Dunnett's test using Graphpad Instat software, *P<0.05, **P<0.01, ***P<0.001 as compared with vehicle-treated animals. The significance level was considered at 2a=0.05. Source: Bhangale et al. (2010).

Table 17. Effect of EtGA leaves on oral glucose tolerance test (OGTT) in diabetic mice. (Bhangale et al., 2010).

Treatment (mg/kg)	Mean fasting glucose level (mg/dl) ± SEM				
	Before Glucose	0 min	30 min	60 min	120 min
Vehicle	402.90±17.44	504.32±15.50	433.44±16.23	388.50±6.69	494.05±14.39
Glyburide (10)	449.93±19.61	525.54±9.91	332.21±16.73***	326.63±4.96***	436.10±19.71
EtGA (100)	476.60±17.73	539.24±15.30	476.23±9.85	395.43±14.71	482.19±18.98
EtGA (200)	465.13±17.88	531.94±15.38	364.73±5.76**	325.42±9.17*	453.63±10.61
EtGA (400)	482.59±14.50	544.26±15.15	349.39±15.96***	297.49±13.05***	480.58±9.07

n = 10, data was analyzed by two-way ANOVA with post hoc Dunnett's test using Graphpad Instat software, *P<0.05, **P<0.01, ***P<0.001 as compared with vehicle-treated animals. The significance level was considered at 2a=0.05. Source: Bhangale et al. (2010).

hypothesized that the late onset of action and prolonged duration of action of EtGA may results from improved pancreatic cytoarchitecture. In this context, other medicinal plants, such as *Cassia auriculata* (Latha and Pari, 2003). *Pleurotus pulmonarius (*Badole et al., 2000) have been reported to possess similar effects. Flavonoids, alkaloids, tannins and phenolics are the other bioactive principles reported to possess antihyperglycaemic activity. ((Kameswararao et al., 1997)). Flavonoids regenerate the damaged ß cells in the alloxan diabetic rats (Chakravarthy et al. 1980).

The traditional medicinal plants with various active principles and properties have been used since ancient times by physicians and laymen to treat a great variety of human diseases such as diabetes, coronary heart disease and cancer. The beneficial multiple activities like manipulating carbohydrate metabolism by various mechanisms, preventing and restoring integrity, function of β-cells, insulin releasing activity, improving glucose uptake and utilization and the antioxidant properties present in medicinal plants offer exciting opportunity to develop them into novel therapeutics. Antihyperglycaemic activity of ethanolic extract of *G. asiatica* may probably be due to the presence of several bioactive antidiabetic principals (Tiwari and Rao, 2002). Before starting the experiment, animals were separated according to their body weight. The animals were injected intraperitoneally (i.p.) at a dose of 150 mg/kg b.w. alloxan monohydrate freshly prepared in normal saline solution. After one hour of alloxan administration, animals were given feed ad libitum and 1ml of (100 mg/ml) glucose i.p. to combat ensuring severe hypoglycemia after 72 hr of alloxan injection; the animals were tested for evidence of diabetes by

Table 18. Effect of the extracts of *Grewia asiatica* (phalsa) leaves on Blood glucose of alloxan diabetic albino rats after acute treatment. (Patil et al., 2010).

Groups (n)	Dose	Blood Glucose level mg/100ml (Mean±SEM)			
		Initial	2nd hour	4th hour	6th hour
Normal control	2 ml saline	106±3.27	107±4.91	107±2.66	104±3.6
Diabetic control	2 ml saline	281±5.03	290 ±4.61	290 ±2.30	292±3.29
Ether extract	200 mg/kg b.w.	298±2.10	277±4.21	248±2.16	241±4.90*
Chloroform extract	200 mg/kg b.w.	305±4.02	255±3.10	205±7.05**	195±5.05**
Alcohol extract	200 mg/kg b.w.	287±5.30	241±2.12	189±3.02**	181±4.06**
Aqueous extract	200 mg/kg b.w.	298±6.08	265±8.20	223±7.05**	210 ±5.61**
Glibenclamide	10 mg/kg b.w	296±4.51	207±1.08	166 ±2.40**	159±1.47**

*p<0.05 – **p<0.01 – compared to Diabetic control. SEM: Standard Error of Mean, n= Number of animals (6). Source: (Patil et al., 2010).

estimating their blood glucose level using glucometer. To the animals, the test extracts (200 mg/kg b.w. orally) and standard drug libenclamide tablets (10 mg/kg b.w. orally) were administered by dissolving in 2% Twen-80/water and normal saline respectively. For acute study, 0.2 ml of blood sample was withdrawn through the tail vein puncture technique using hypodermic needle at interval of 0, 2nd, 4th and 6th h of administration of single oral dose. The animals were segregated into seven groups of six rats each for each extract. For all drugs groups of normal, diabetic control and standard glibenclamide were kept same for comparison with ether, chloroform, alcohol and aqueous extracts of drugs. The mean ± SEM were statistically calculated for each parameter (Kulkarni, 1999).

The results of effect of extracts leaves of *Grewia asiatica* (Phalsa) which are expressed as change in blood glucose level are shown in Table 18 (Patil et al., 2010). More significant (p<0.01) anti-diabetic activity was observed in alcoholic and chloroform extracts of Grewia *asiatica* (Phalsa) in acute model compared with standard glibenclamide. Ether extract of Grewia *asiatica* (Phalsa) has not shown significant anti-diabetic activity (p<0.01) in acute study. In vivo efficiency was performed in healthy normal Wistar rats by measuring the hypoglycemic effect produced after oral administration. (Kahn and Shechter, 1991) have suggested that a 25% reduction in blood glucose levels is considered a significant hypoglycemic effect. The results of the study were satisfactory and revealed that the alcoholic and chloroform extracts of leaves of Grewia *asiatica* (Phalsa) has exhibited significant hypoglycemic activity. The reduction of blood glucose level in alloxan induced rat was found highest in alcoholic and chloroform extracts of *G. asiatica* (Phalsa).

Hypoglycemic activity

Leaves

Patil et al., (2011) reported different extract of *G. asiatica* leaves for their hypoglycemic activity on alloxan induced diabetic wister rats. Ethanol extracts (200 mg/kg) showed more significant reduction in blood glucose level in alloxan induced diabetic Wister rats in comparison to control and the standard drug, glibenclamide. Aqueous extracts of leaves were administered orally (250 and 500 mg/kg) to normal rats and streptozotocin (STZ) (50 mg/kg) treated diabetic rats. Administration of extracts for 21 days significantly reduced blood glucose level in STZ induced diabetic rats. The plant extract was evaluated by oral glucose tolerance test model for its influence at different doses on blood glucose levels in normal rats fed with overload of glucose. Extracts significantly reduced the blood glucose level in a dose dependant manner. The results suggested that aqueous extracts significantly increased the glucose tolerance in normal rats (Latif et al., 2012).

Effect on glycemic index

The glycemic index (GI) of a food is defined as the area under the two hour blood glucose response curve (AUC) following the ingestion of a fixed portion (usually 50 g) (Frost et al., 1999). For GI calculation, the AUC of the test food (that is, phalsa fruit extract) is divided by the AUC of the standard (Glucose) and multiplied by 100 (Salmeron et al., 1997)

Fruit

Table 19 (Mesaik et al., 2013) showed comparative PGL in fasting as well as at 60, 120, and 180 min after test meal consumption by the GG, GPG, and PG groups. Compared to fasting plasma glucose level (PGL), consumption of D-glucose resulted in 90 and 34% increase in PGL at 30 and 120 min respectively. However, considerably decreased PGL was recorded after 120 min (20%) showing typical glucose tolerance phenomenon. On the other hand, consumption of a

Table 19. Plasma glucose levels (PGL) of subjects before and after consumption of D-glucose (GG), a mixture of phalsa fruit extract+D-glucose (GPG), and phalsa fruit (PG). Percentage increase or decrease in PGLs indicated within parentheses; change (increase/decrease) in the PGL is shown by arrows. (Mesaik et al., 2013)

Test/Time	0	30 min	90 min	120 min
GG	69.6	130.2 (89.5%↑)	92.1 (34.1%↑)	69.7 (1.4%↑)
GPG	64.7	124.2 (80.7%↑)	83.5 (21.5%↑)	81.9 19.2%↑)
PG	72	69.7 (1.4%↑)	67.7 (1.4%↓)	73.1 (6.4%↑)

Source: Mesaik et al. (2013).

Table 20. Effect of the Extracts of *Grewia asiatica* (Phalsa) *Leaves* on blood glucose of alloxan diabetic albino rats after acute treatment. (Patil et al., 2011)

Group (n)	Serum Glucose Level (mg/dl) (Mean ±SEM)					
	0 h	1 h	2 h	3 h	5 h	7 h
Normal	138.25±5.45	139.75±5.58	138.25 ±4.58	139.56 ± 4.57	137.56± 4.5	139.75±3.12
Control (diabetic)	220.45±6.88	221.34±5.78	223.43±5.84	224.65±2.82	225±5.83	227.3±5.94
Standard (10 mg/kg)	221.07± 5.95	217.22±4.69	211.07±4.70	*208±2.71	*205.77±4.01	**201.08
Pet. Ether (200 mg/kg)	224.54±4.36	223.77±4.21	220±4.19	218.44±2.51	217.65±4.70	216.05±5.75
Chloroform (200 mg/kg)	223.66±5.91	220.53±4.08	219.35±5.04	217.89±2.62	215.38±3.68	214.4±4.80
Ethanol (200 mg/kg)	222.08±5.99	218.43±5.46	214.32±4.30	211.76±3.56	*209.89±5.19	**205.43±7.1

p<0.05 – Significant, **p<0.01 - More Significant Compare to Control, SEM: Standard Error Mean, n = Number of Animals (6). Source: Patil et al. (2011).

mixture of glucose and phalsa fruit extract (GPG) resulted comparably low PGL after 30 min (80%), 90 min (21%) and 120 min (6.4%). Hence, phalsa fruit exhibited modest hypoglycemic effect. The present data are, for the most part, consistent with previous studies on glycemic response of phalsa. The glycemic index of phalsa fruit was also estimated using the PGL after intake of phalsa fruit (PG) (Mesaik et al., 2013). The low glycemic index of phalsa fruit indicated that ingestion of fruit would not elevate the plasma glucose level in spite of the sweat taste. Phalsa fruit extract has previously reported to affect glucose metabolism and immune system (Sisodia and Singh, 2009).

Leaves

The Preliminary phytochemical study performed revealed the petroleum ether extract consist diterpines, glycosides, fats, chloroform extract consist alkaloids, glycosides and Ethanol extract consist triterpenoids, sterols flavonoids, saponins, tannins as active principal. In Evaluations of hypoglycemic activity in acute as well sub acute study Ethanolic extract of Phalsa leaves showed significant decrease in serum glucose level, while petroleum ether and chloroform extract did not show significant decrease in serum glucose level as compared to standard drug (Glibenclamide). The results are given in Tables 20 and 21 (Patil et al., 2011). This *in vivo* study demonstrated significant hypoglycaemic activity was found highest in ethanolic extract of *G. asiatica* (Phalsa) leaves.

Bark

The bark of *G. asiatica* (Phalsa) plant showed hypoglycemic effect on alloxan diabetic male rabbits (Dogar et al., 1998).

Antipyretic activity and analgesic activity

Fruits

G. asiatica fruits has been evaluated as potent analgesic and antipyretic agent by taking various doses of aqueous extract of its, by comparing with the standard drug, i.e. aspirin. The results, verified by statistical analyses (‘t’ test and confident level), shown in Table 22 (Das et al., 2012). Analgesic activity of *G. asiatica* was observed in 5 animal sets and antipyretic activity in one animal set. Analgesic activity was observed by writhing test 12 on administrating acetic acid (0.6%) in the dose of 10ml/kg, inducing pain (Koster et al., 1959). The standard drug, aspirin was used in the dose of 400 mg/kg, to arrest the acetic acid induced pain. From the findings it is appeared that *G. asiatica* in the dose of 100 mg to 250 mg/kg body weight showed significant inhibitory effect on acetic acid induced pain. 300 mg/kg body weight of *G. asiatica* has also shown good inhibitory effect which is similar to aspirin.

Table 21. Effect of the Extracts of *Grewia asiatica* (Phalsa) *Leaves* on blood glucose of alloxan diabetic albino rats after Sub acute treatment. (Patil et al., 2011)

Group (n)	Serum Glucose Level (mg/dl) (Mean ± SEM)					
	0 day	1 day	2 day	3 day	5 day	7 day
Normal	139.25±3.45	137.45±5.67	139.45±6.45	137.75±5.89	138.65±3.58	139.55±5.45
Control (diabetic)	220.33±1.88	225±5.83	226.75±2.84	222.44±2.82	214. 25±5.85	209±6.94
Standard (10 mg/kg)	221 .25±1.95	205.77±4.09	196.25*±2.70	184.43*±2.71	173**±6.01	162.66**
Pet. Ether (200 mg/kg)	224±2.36	217.65±4.70	211.50±3.19	209. 89±2.51	204 .75±1.70	201.25±6.75
Chloroform (20 0mg/kg)	223 .05±2..91	215.38±3.68	210.65±2.04	206.05±2.62	202 .09±0.68	199.67±5.80
Ethanol (200 mg/kg)	222.08±1.99	208.89±5.91	199.25±5.30	187*±4.56	180.78**±3.19	172.75**±7..81

*p<0.05 – Significant, **p<0.01 - More Significant Compare to Control, SEM : Standard Error Mean, n = Number of Animals(6). Source: Patil et al. (2011).

Table 22. Effect of Grewia asiatica (Phalsa) on acetic acid induced pain (writhing test). (Das et al., 2012).

Sample	Average# number of writhing ± SE (30 minute)					
	A1	A2	A3	A4	A5	Average
Group I	9.00±0.707	10.20±0.43	10.0±0 0.7	11.00±0.71	10.67±0.89	2.56±1.14
Group II	8.20±0.8	8.20±0.86	8.24±0.8	9.4±0.81	8.24±0.76	3.08±1.37
Group III	5.60±0.6	4.8±0.80	5.00±0.44	5.28±0.56	4.2±0.48	3.64±1.32
Group IV	2.40±0.40	2.4±0.5	2.6±0.4	2.2±0.48	2.00±0.21	1.66±0.74
Group V	0.00±0.00	0.2±0.08	0.2±0.08	0.2±0.08	0.00±0.00	0.55±0.25
Asperin	0.4±0.4	0.2±0.08	0.00±0.00	0.2±0.08	0.00±0.00	0.88±0.39
Acetic acid	19.00±1.7	20.4±1.806	19.26±0.47	20.4±1.05	21.60±2.23	20.17±0.34

Group-I to Group-V indicate G. asiatica in the dose of 100 mg, 150 mg, 200 mg, 250 mg and 300 mg per kg body weight respectively. Aspirin used in the dose of 400 mg/kg body weight. A1 - A5 indicate number of experimental animals. # Average (number of writhing) of 5 animals, SE = Standard error (n = 5). Source: Das et al. (2012).

Antimalarial and antiemetic activities

Fruits

Yaqueen et al., (2008) reported the evaluation of the antiemetic activities of alcoholic extracts of fruits of *G. asiatica* (Phalsa) in dog, whereas acute oral toxicity test was carried out in mice and rats. Oral dose of 200 and 600 mg/kg of a crude alcoholic extract was found non-toxic in mice and rats. Oral dose of crude alcoholic extract (120 mg/kg body weight) caused antiemetic effect in dogs in 3 h and controlled emesis centrally induced by apomorphine (0.044 mg/kg body weight).

This activity was comparable to standard commercial anti-emetic drugs like Maxolon (metoclopramide) and Largactil (chlorpromazine).

Leaves

Zia-Ul-Haq et al. (2012) reported the antimalarial and antiemetic activities of methanolic extract of leaves. The study indicated that *G. asiatica* leaves are a potential source of antimalarial and antiemetic drugs. The crude methanol extract showed antimalarial activity, (69% inhibition). Methanolic extract was administered to male

chicks at 50 and 100 mg/kg dose levels and percent inhibition of emetic action was 39.14 and 59.69%, respectively.

Antiplatelet activity

Leaves

There is a great interest in exploring the anti-platelet activity of medicinal plant extracts because these are inexpensive and easily available from indigenous resources. Zia-Ul-Haq et al. (2012), reported the anti-platelet activity of a crude methanol extract of *G. asiatica* L. leaves. The extract exhibited a potent platelet aggregation inhibition activity, in a dose-dependent manner at a concentration range of 1 to 10 mg/m, suggesting that this extract can be considered as treatment for prevention of cardiovascular or inflammatory diseases.

Anti-hyperlipidemic activity

Leaves

Leaves were investigated for their anti-hyperlipidemic activity in induced-hyperlipidemic rats. The data suggested that extract had potent anti-hyperlipidemic effects. Fifty compounds were identified, of which six triterpenes, two sterols, one diterpene and four fatty alcohols were isolated. However, it has not been established which compound is responsible for the anti-hyperlipidemic effects (Abou Zeid and Sleem, 2005).

Antiviral activity

Leaves

Sangita et al. (2009) reported the antiviral activity of an extract of *G. asiatica* leaves against Urdbean leaf crinkle virus (ULCV). The test plants previously sprayed with 500, 1,000, 1,500 and 2,000 µg/mL of *G. asiatica* extract were recorded as 58, 34, 38 and 48% of virus infection, respectively, in comparison to 90% infection of control. It was found the maximum inhibitory activity at 1,000 µg/ml and fairly good activity at concentrations of 1,500 and 2,000 µg/ml.

Anti-Inflammatory activity

Fruits

A methanol extract of fruit of *G. asiaica* was screened for its possible anti-inflammatory activity on carrageenan induced edema in rat paw at doses of 250 and 500 mg/kg, orally. The extract showed significant ($p < 0.001$ and $p < 0.01$) anti-inflammatory activity at both doses (Bajpai et al., 2012).

Antifertility

Seeds

Seeds of *G. asiatica* has been used as antifertility agent and was reported to have anti-implantation and abortifacient activities (Pokharkar et al., 2010)

Conclusion

Grewia asiatica (Phalsa) is a food plant and can also be used as an herbal medicine for the treatment of various diseases. In traditional folklore medicine, the fruit has been used as astringent, stomachic and cooling agent. When unripe, it has been reported to alleviate inflammation and was administered in respiratory, cardiac and blood disorders, as well as in fever. Root and bark has been prescribed for rheumatism and its infusion used as a demulcent. The leaves were applied on skin eruptions. Number of therapeutic research carried out on different part of this plant like fruits, leaves, stem etc. The plant has been reported to possess antioxidant, antidiabetic, antihyperglycaemic, radioprotective, antimicrobial, hepatoprotective, antifertility, antifungal, antilagesic, antipyeretic and antiviral activities. Above mentioned studies mostly addressed the basic chemical and pharmacological characteristics of phalsa fruit. Detailed studies are needed for identifying and quantifying bioactive constituents of every part of plant responsible for tagged activities. There is need to develop new varieties with big fruits having greater yield, improved quality, sweetness, flavor and which can be grown in colder regions also.

Conflict of Interest

The authors have not declared any conflict of interest.

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Anticoagulant and anti-inflammatory activity of a triterpene from *Protorhus longifolia* stem bark

Rebamang A. Mosa*, Thabo Ndwandwe, Nontando F. Cele and Andy R. Opoku

Department of Biochemistry and Microbiology, University of Zululand, Private Bag X1001, KwaDlangezwa 3886, Republic of South Africa.

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This work evaluated the anticoagulant and anti-inflammatory activity of a lanosteryl triterpene (3β-hydroxylanosta-9,24-dien-21-oic acid) isolated from *Protorhus longifolia* stem bark. Tail bleeding time assay was used to investigate the *ex vivo* anticoagulant activity. The effect of the triterpene on the thermally induced aggregation of malate dehydrogenase (MDH) and citrate synthase (CS) was studied. The anti-inflammatory activity of the triterpene was investigated using the cotton pellet-induced granuloma model in rats. Granuloma formation was measured following 7 days of oral administration of the experimental rats (two groups) with the triterpene at 50 and 250 mg/kg body weight (b.w). The compound (50 mg/kg) significantly ($p < 0.05$) increased bleeding time in rats by up to 7 min as compared to 2.5 min observed in the normal control group. It also improved the activity of Hsp70 on MDH and CS aggregation suppression. The reduction of the granuloma formation by up to 40.3% was observed. It is apparent that the triterpene has potential to inhibit the aggregation of proteins.

Key words: Triterpene, anti-inflammatory activity, *Protorhus longifolia,* protein aggregation.

INTRODUCTION

Thromboembolic disorders are the leading cause of stroke, myocardial infarction, and pulmonary embolism. These disorders contribute to over 20% of annual deaths globally (Cohen et al., 2011). There is an established link between blood coagulation and inflammation (Verhamme and Hoylaerts, 2009; Chu, 2011). This linkage is observed under both pathophysiological and physiological conditions. Upon endothelial injury, inflammation and blood coagulation become autocatalytic. Inflammation suppresses the natural anticoagulant system, which favors the expression of prothrombogenic molecules (Verhamme and Hoylaerts, 2009). Furthermore, inflammation has also been linked with most chronic illnesses including diabetes and cardiovascular diseases (Aggarwal et al., 2006). Anticoagulants and anti-inflammatory compounds are thus, important in the management of thromboembolic disorders (Hirsh et al., 2007). The reported (Khanapure et

*Corresponding author. E-mail: rebamang@gmail.com, Tel: +2735 9026099. Fax: +2735 9026568.

al., 2007; Mavrakanas et al., 2011) undesirable side-effects of the current anticoagulant and anti-inflammatory agents have stimulated the search for new generation of more effective agents from natural sources.

Medicinal plants are reservoirs of bioactive compounds vital to human health. Their extracts provide a useful source of biologically active compounds which can be used as novel structural templates or be directly developed as active agents (Kee et al., 2008). Natural products identified from traditional medicinal plants have become very important in the development of therapeutic agents. Triterpenes are a group of biologically active plant secondary metabolites predominantly found on plant surfaces such as stem bark and leaves (Jäger et al., 2009). These compounds have been reported to exhibit potentially significant pharmacological effects such as anti-platelet aggregation (Mosa et al., 2010a), anti-hyperglycaemic (Ghosh et al., 2011), anti-hyperlipidemic (Liu et al., 2007), and anti-inflammatory activities (Yadav et al., 2010). Plant derived triterpenes have thus become new targets for drug development.

Protorhus longifolia (Benrh.) Engl. (Anacardiaceae) is an evergreen tall (about 15 m) tree indigenous to Southern Africa. In the genus *Protorhus, P. longifolia* is the only species that is found in Southern Africa (Archer, 2000). The bark of this plant is traditionally used to cure various ailments. Among its many known traditional uses is its use to “thin” blood. Anti-platelet aggregation activity of the crude extracts as well as the lanosteryl triterpenes from of *P. longifolia* stem bark have been reported (Mosa et al., 2011a, b).

As part of our on-going evaluation of some bioactivity of the lanostane-type of triterpenoid (3β-hydroxylanosta-9,24-dien-21-oic acid) from *P. longifolia*, we report, in this work, the *ex vivo* anticoagulant and anti-inflammatory activity of this triterpene.

MATERIALS AND METHODS

Reagents and chemicals

Unless otherwise stated, the reagents and chemicals were purchased from Sigma-Aldrich (St Louis, USA).

Plant

Plant material (fresh stem barks) of *Protorhus longifolia* was collected from Hlabisa, KwaZulu-Natal, South Africa. The plant (voucher specimen number RA01UZ) was authenticated by Dr. N.R. Ntuli, Department of Botany, Zululand University. The plant material was air-dried, powdered and stored at 4°C until processing.

Extraction and isolation

The method of extraction and isolation of the triterpene from *P. longifolia* stem bark have been previously described (Mosa et al., 2011a; Mosa et al., 2014). Briefly, the plant material was first defatted with *n*-hexane and then extracted with chloroform. The triterpene was isolated from the chloroform extract (13 g) over column chromatography (24 × 700 mm; Silica gel 60; 70-230 mesh ASTM; Merck, Darmstadt, Germany), eluted with *n*-hexane-ethyl acetate solvent system (9:1 to 3:7, gradient). The collected fractions (20 ml) were analysed with thin layer chromatography (TLC aluminium sheets, F_{254}). Structure of the compound (RA5, 0.72 g) was confirmed using spectroscopic techniques. Melting point was determined using Stuart SMP 11 melting point apparatus (Shalom Instruments supplies, Durban, South Africa).

Malate dehydrogenase (MDH) and citrate synthase (CS) aggregation suppression assay

The assay was conducted by evaluating the ability of the triterpene to suppress MDH and CS thermally induced aggregation. The heat stability of the protein and the triterpene was assessed following the method described by Ramya et al. (2006) with some modifications. Heat shock protein 70 (Hsp70, Human recombinant) and the triterpene (100 µl each) were separately prepared in the assay buffer (100 mM NaCl, 20 mM Tris, pH 7.4) at a final concentration of 1.3 µM. Turbid metric changes at 48°C were then followed at 340 nm for 45 min with Biotek plate reader (ELx 808 UI, Biotek Instrument Supplies) equipped with Gen5 software. The effect of the triterpene to prevent thermally induced aggregation of MDH and CS at 48°C was conducted as described for heat stability assessment (Shonhai et al., 2008). MDH and CS (each at 1.3 µM) were separately incubated with the Hsp70 (1.3 µM) in the presence and absence of the triterpene at 1.3 and 5.2 µM. The thermally-induced aggregation of MDH and CS at 48°C was then separately followed by reading turbidity changes at 340 nm for 45 min. For control experiments, aggregation of MDH alone, aggregation of MDH in the presence of 1.3 µM bovine serum albumin (BSA), and aggregation of MDH in the presence of Hsp70, was separately assessed. Similar controls were used for CS and the experiments were replicated three times.

Animals

Approval (UZREC 171110-030 Dept 2012/23) for use of animals and experimental protocols was issued by the Institutional (Zululand University) Research Animal Ethics Committee. Adult Sprague-Dawley rats (7 to 9 weeks old, 220 ± 20 g) of either sex were obtained from the Department of Biochemistry and Microbiology animal house, Zululand University. The rats were maintained under standard conditions (12 h light-dark cycle at 23 ± 2°C); they had free access to enough drinking water and standard pellet rat feed.

Anticoagulant activity (*ex vivo*)

Tail bleeding time assay

Tail bleeding time assay described by Gadi et al. (2009) was adapted with some modification to evaluate the anticoagulant activity of the triterpene. The adult Sprague-Dawley rats (220 ± 20 g) were randomly divided into four groups of five rats per group. Two groups of the rats were administered (orally) with different concentrations (50 and 250 mg/kg body weight- b.w) of the triterpene 2 h before experiment. Positive and negative control

groups were administered with acetylsalicylic acid (ASA) (30 mg/kg b.w) and 2% Tween 20, respectively. The rats were slightly anaesthetized. Bleeding time was measured by cutting off the rat's tail tip (5 mm) and blood was blotted on a filter paper at 30 s interval until no blood was observed on the filter paper. The period between the tail amputation and the cessation of bleeding was taken as the bleeding time in minutes.

Platelet preparation

After the bleeding time has been determined, blood samples from the rats in the respective groups were collected to obtain platelets. The animals (rats) were killed by a knock on the head and this was followed by immediate collection of the blood from abdominal aorta. The blood collected was separately mixed with an acid-dextrose-anticoagulant (0.085 M trisodium citrate, 0.065 M citric acid, 2% dextrose). The platelets were obtained through successive centrifugations followed by washing of the blood (Tomita et al., 1983). The obtained platelets were further suspended in a buffer (resuspending buffer, pH 7.4).

Ex vivo anti-platelet aggregation

The *ex vivo* anti-platelet aggregation activity of the triterpene was determined on thrombin-induced platelet aggregation (Mekhfi et al., 2004). The platelets (200 µl) from the different groups were separately pre-incubated in a 96-well micro plate for 5 min at 37°C before addition of thrombin (20 µl, 5 U/ml). The reaction was monitored by reading absorbance at 415 nm (Biotek plate reader, ELx 808 UI) for 20 min at 30 s interval. The experiment was replicated three times and the mean slope (A) ± SD was reported. Percentage (%) inhibition of platelet aggregation was calculated from the given formula:

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0 \times 100]$$

Where, A_0 represent the mean slope of control and A_1 represent the mean slope of the test drug.

Cotton pellet-induced granuloma

The anti-inflammatory activity of the triterpene was investigated using cotton pellet-induced granuloma model (Penn and Ashford, 1963). The rats (220 ± 20 g) were divided (randomly) into four groups of five rats per group. The animals were orally pre-administered with the drugs 30 min before interscapular implantation (under slight anaesthesia) of pre-weighed sterile cotton pellets (20 mg). The experimental groups (I and II) were administered with the triterpene at 50 and 250 mg/kg b.w, respectively, dissolved in 2% Tween 20. The positive and negative control groups (III and IV) were administered with 10 mg/kg b.w of indomethacin and 2% Tween 20, respectively. The rats were orally administered with the drugs for seven consecutive days. On the eighth day, the rats were slightly anaesthetised and the cotton pellets were carefully removed without the extraneous tissue. The wet pellets were weighed and dried at 37°C for 24 h. The increment in weight of the dry pellets was considered as a measure of granuloma formation. The anti-proliferative activity of the triterpene was compared with that of the control. Percentage inhibition of granuloma formation was calculated from the formula:

$$\% \text{ Inhibition} = (W_c - W_t/W_c) \times 100$$

Where, W_C represents the pellet weight from the animals in the control group and W_t represents the pellet weight from the animals in the drug-treated group.

Biochemical estimation

The method previously described by Nagar et al. (2011) was adapted with modifications to prepare tissue homogenate. The granuloma tissue was homogenized (10 ml/g) in cold Tris-HCl buffer (0.1 M, pH 7.8, containing ethylene diaminetetraacetic acid - EDTA). This was followed by centrifugation at 10,000 × g for 15 min at 4°C. The supernatant was collected and kept on ice. The supernatant was used for estimation of protein content, catalase and superoxide dismutase (SOD) activities.

Protein content

The Bradford assay was used to determine protein content of the homogenate. Coomassie Brilliant Blue G-250 (5 ml) was added to 100 µl of the diluted (1:10) homogenate. The mixture was incubated for 5 min. BSA at concentrations ranging from 5 to 100 µg/100 µl normal saline, was used as a standard. The Biotek plate reader was used to read absorbance at 595 nm. Protein concentration was determined from a calibration curve.

Catalase activity

Catalase activity was estimated by measuring H_2O_2 decomposition (Aebi, 1983). The reaction mixture consisted of the homogenate (20 µl) and 2 µl of phosphate buffer (0.1 M, pH 7.2). The reaction was then initiated by adding 250 µl of 30 mM H_2O_2. The change in absorbance was monitored at 240 nm with UV-Vis spectrophotometer for 3 min at 30 s interval. The results were expressed as H_2O_2 decomposed/minute/mg protein, using 43.6 as the molar extinction coefficient of H_2O_2.

Superoxide dismutase activity

The nitroblue tetrazolium (NBT) reaction method (Glannopolittis and Ries, 1977) was followed to estimate the superoxide dismutase activity. The reaction mixture contained 100 µl of homogenate, 0.4 ml of 0.01% NBT, 1 ml of 0.05 M sodium carbonate, and 0.2 ml of 1 mM EDTA. Initial absorbance (zero minute) was read at 630 nm and this was followed by addition of 0.4 ml of 2.4 mM hydroxylamine hydrochloride to initiate the reaction. The reaction mixture was then incubated at 25°C for 5 min. The NBT reduction was measured at 630 nm (Biotek plate reader, ELx 808 UI) and one enzymatic unit of SOD was taken as the amount of protein (in the form of enzyme) present in 100 µl of homogenate. The enzymatic activity of SOD was expressed as unit/mg protein.

Data analysis

The results were presented as mean ± SEM. One way analysis of variance (ANOVA) followed by Tukey multiple comparison test, were used to assess statistical differences. Where the *p* value is less than 0.05 the results were taken to be statistically significant.

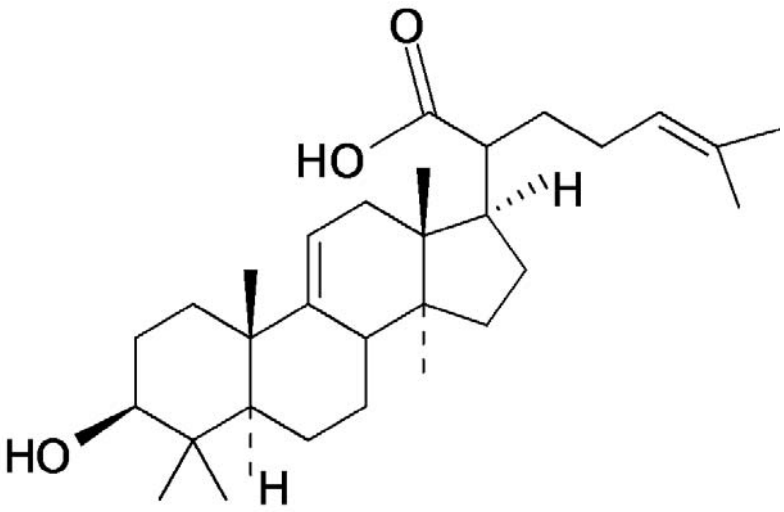

Figure 1. Chemical structure of 3β-hydroxylanosta-9,24-dien-21-oic acid (RA5).

RESULTS AND DISCUSSION

The lanosteryl triterpene, 3β-hydroxylanosta-9,24-dien-21-oic acid (RA5, Figure 1) has previously been isolated and characterized from *P. longifolia* stem bark (Mosa et al., 2011a; Mosa et al., 2014). Even though inappropriate protein aggregation is normally prevented by complex cellular mechanisms, however under certain circumstances aggregation of proteins occurs. Misfolding and inappropriate aggregation of proteins is commonly associated with a number of neurodegenerative diseases (Lendel et al., 2009). Proteins are known to aggregate in response to heat stress. The thermal aggregation of MDH and CS, and the effect of Hsp70 on their aggregation are well documented (Shonhai et al., 2008). Hsps are ubiquitous proteins found in the cells of all living organisms. These proteins function as intra-cellular chaperones for other proteins, preventing protein misfolding and unwanted protein aggregation (Shonhai et al., 2008). Hsps are involved in many disease processes, including cardiovascular diseases (Whitley et al., 1999). Therefore, there is a growing interest in discovery of pharmacologically active drugs that up regulate expression of Hsps as a potential therapeutic effect in human disease.

Since blood coagulation and inflammation are both mediated by activation and aggregation of proteins, it was appropriate to evaluate the effect of RA5 (triterpene) on the MDH/CS/ Hsp70 system. Figures 2 and 3 indicate that, like the Hsp70, the triterpene suppressed the thermally induced aggregation of MDH and CS. The triterpene (1.3 and 5.2 µM) also improved the ability of Hsp70 to suppress aggregation of the proteins. The effect of the triterpene was concentration dependent. The ability of the compound to improve the activity of Hsp70 suggests that the triterpene could partly be exerting its therapeutic properties through inhibition of protein aggregation.

The *in vitro* anticoagulant and/or anti-platelet aggregation activity of some plant-derived triterpenoids have been reported (Habila et al., 2011; Lee et al., 2012). The ability of RA5 to inhibit the aggregation of platelets induced by thrombin and other clotting agents has also been reported (Mosa et al., 2011a). The *ex vivo* anticoagulant activity of the triterpene was assessed using tail bleeding time assay. It is apparent that the triterpene significantly ($p < 0.05$) prolonged the tail bleeding time (Table 1) and thus exhibits anticoagulant properties. The anti-platelet aggregation activity of the triterpene was however, less than that of ASA - a standard anti-platelet agent. The lower activity relative to the standard drug could be due to the bioavailability of the compound. Furthermore, previous studies (Mekhfi et al., 2008; Gadi et al., 2009) have demonstrated that there is not always a direct correlation between inhibition of platelet aggregation and the bleeding time. Also, the possibility that ASA could have permanently inhibited activation and aggregation of the platelets could not be ruled out in contributing to the higher anti-platelet aggregation activity in the positive control.

In our previous study (Mosa et al., 2011a) we reported the anti-inflammatory activity of RA5 using the carrageenan-induced paw edema model which is used for acute inflammation. In this study, the anti-proliferative

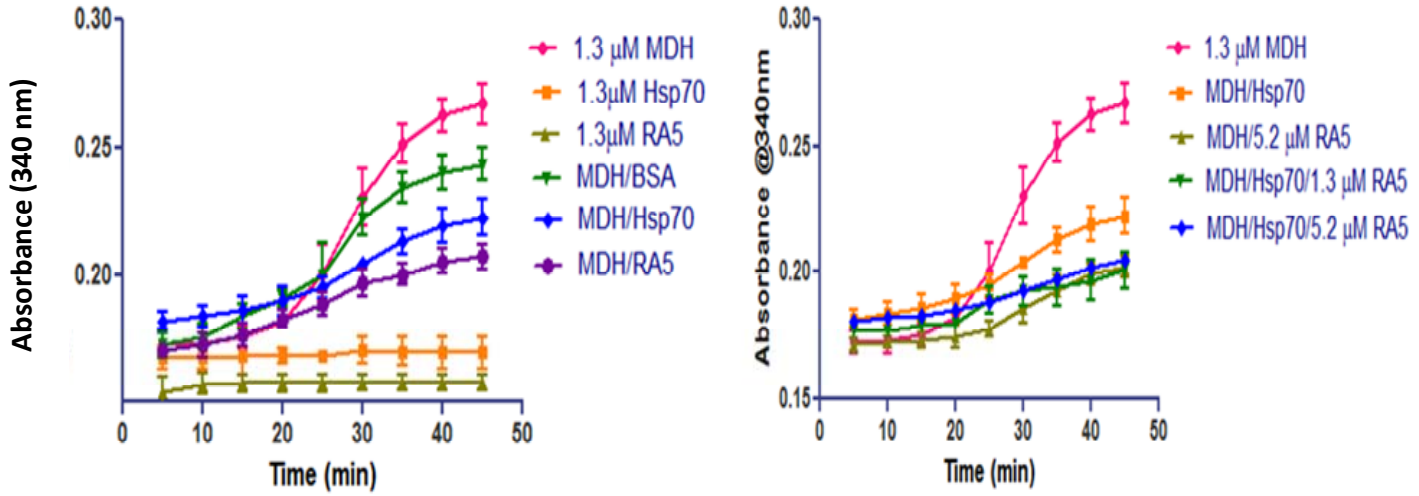

Figure 2. Effect of **RA5** (1.3 and 5.2 µM) on thermally induced aggregation of MDH at 48°C. The aggregation of MDH (1.3 µM) was induced in the absence and presence of the triterpene at different concentrations. For control experiments, aggregation of MDH alone, MDH aggregation in the presence BSA, and MDH aggregation in the presence of Hsp70 were separately assessed. Data were expressed as Mean ± SEM.

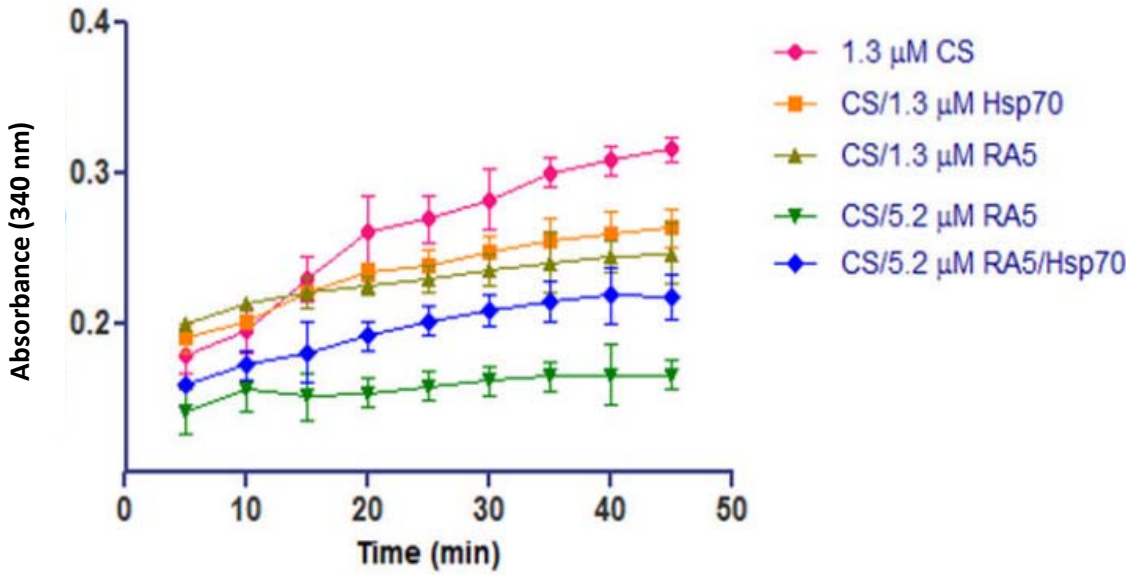

Figure 3. Effect of **RA5** on thermally induced aggregation of CS at 48°C. CS (1.3 µM) was exposed to heat stress in the absence and presence of the triterpene (1.3 and 5.2 µM). Data were expressed as Mean ± SEM

activity of the triterpene was investigated using the cotton pellet-induced granuloma model. Chronic inflammation is characterized by infiltration of mononuclear cells, fibroblast proliferation and increased connective tissue formation (Beni et al., 2011). Cotton pellet-induced granuloma model is commonly used as a suitable *in vivo* test for chronic inflammation. The weight of wet and dry cotton pellets correlates with the amount of formed transudate material and granuloma tissue, respectively. The triterpene (50 and 250 mg/kg b.w) significantly ($p < 0.05$) decreased transudates and granuloma formation in the rats (Table 2). The inhibitory effect of the compound compared favorably with that of the indomethacin (10 mg/kg), a known non-steroidal anti-inflammatory drug

Table 1. Effect of the RA5 on tail bleeding time and inhibition of platelet aggregation.

Parameter	Control	RA5		ASA
Dose	**2% Tween 20**	**50 mg/kg**	**250 mg/kg**	**30 mg/kg**
Bleeding time (min)	2.50	7.00 ± 1.04*	6.00 ± 0.79*	4.50 ± 0.46*
Antiplatelet aggregation (%)	0.00	4.53	9.32	19.3

Values are expressed as mean ± SEM, n = 5. *p < 0.05 compared to control

Table 2. Effect of RA5 on transudative and granuloma formation.

Group	Dose mg/kg	Granuloma wet weight (mg)	Granuloma dry weight (mg)	Transudative weight (mg)	Granuloma inhibition (%)
Control	-	338 ± 4.05	96.6 ± 0.51	241.4 ± 4.33	-
Indomethacin	10	168 ± 2.43*	65.9 ± 2.43*	102.1 ± 2.33*	31.8
RA5:	50	222 ± 2.90*	67.9 ± 0.76*	154.1 ± 1.42*, †	29.7
	250	152 ± 2.15*, †	57.7 ± 0.81*, †	94.3 ± 3.18*, †	40.3

Values are expressed as mean ± SEM, n = 5. * p < 0.05 compared to control, † p < 0.05 compared to 50 mg/kg.

Table 3. Effect of RA5 on protein content, catalase and SOD activity on the cotton pellet-induced inflammation in rats.

Group	Dose (mg/kg)	Protein content (mg/ml)	Catalase activity (µmol H_2O_2 decomposed/min/mg protein)	SOD activity (units/mg protein)
Control	-	0.68 ± 0.11	96.7 ± 2.88	0.75 ± 0.42
Indomethacin	10	0.56 ± 0.19	119.1 ± 1.01	1.26 ± 0.75
RA5	50	0.48 ± 0.94	242.5 ± 0.83*,†	1.04 ± 0.09
	250	0.52 ± 0.07	256.4 ± 1.22*, †	1.08 ± 0.26

Values are expressed as mean ± SD, n = 5. * p < 0.05 compared to control, † p < 0.05 compared to indomethacin

(NSAID). The anti-proliferative activity of NSAIDs is characterized by decrease in granuloma tissue formation, collagen fibre generation, and suppression of muccopolysaccharides (Verma et al., 2010). The results from this study suggest the NSAIDs character of the triterpene from *P. longifolia* which indicates its potential to prevent chronic inflammation. The anti-inflammatory effect of other triterpenes has been previously reported (Yadav et al., 2010).

Inflammation is associated with excess production of superoxide radicals (Chang et al., 2010) which intensify inflammation by stimulating secretion of inflammatory mediators from macrophages (Porfire et al., 2009). Endogenous antioxidant enzymes such as SOD and catalase help to destroy the free radicals. Table 3 shows that the triterpene did not only reduce the protein content in the granulation tissue but also significantly increased the activity of catalase ($p < 0.05$) and SOD as compared to the control group. These results partly explain a relatively higher (40.3%) inhibitory activity of the triterpene (250 mg/kg) observed on the granuloma formation. It is apparent that the ability of the compound to increase activity of SOD and catalase is vital in the compound's anti-inflammatory activity.

Conclusion

Platelets play a central role in linking inflammation and blood coagulation. Activated platelets release pro-inflammatory mediators and cytokines (Blair and Flaumenhaft, 2009). Consequently, inflammation suppresses the natural anticoagulant system, which favors the expression of prothrombogenic molecules

(Verhamme and Hoylaerts, 2009). Anti-inflammatory compounds may significantly prevent inappropriate blood coagulation and therefore alleviate thromboembolic disorders. This study demonstrated that the triterpene (3β-hydroxylanosta-9,24-dien-21-oic acid) from stem bark of *P. longifolia* possesses anticoagulant and anti-inflammatory activity. The observed activities of the triterpene could be attributed to its ability to inhibit protein aggregation. Further work is required to evaluate the effect of this compound on the Hsp70 expression.

Conflict of Interest

The authors have not declared any conflict of interest.

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