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Research Article

COMPARATIVE ANALYSIS OF TOTAL FLAVONOIDS, QUERCETIN CONTENT AND ANTIOXIDANT ACTIVITY OF *IN VIVO* AND *IN VITRO* PLANT PARTS OF *GREWIA ASIATICA* MAST

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ABSTRACT

The present investigation deals with the comparison of total flavonoids, Quercetin content and antioxidant activity of *in vitro* and *in vivo* parts of *Grewia asiatica* Mast. (Phalsa, Tiliaceae). Due to the presence of active metabolites the plant is supposed to have high antioxidant activity that may be the reason of high medicinal potential of plant. For investigation of *in vitro* plants, callus culture was made. Callus from leaf discs and nodal explants were best induced on MS media with NAA (0.5 mg/lt) and established on various concentrations of BAP. The flavonoid contents in the *in vivo* (leaf, stem) and *in vitro* (old callus) plant parts were found to be present in the alcoholic, chloroform and aqueous solvents. Highest concentration of total flavonoids was found in the ethanolic solvent of leaf in spectrophotometric assay. Through the HPLC analysis revealed that the amount of Quercetin in the leaf sample was found to be double that of the callus (4.28ng/µl). Antioxidant potential of the fruit extract was observed maximum (98.2%) among all plant parts. For the stem extract it was found to be higher (89.8%) than leaf and callus by the DPPH assay. The present investigation revealed that the leaves, stem and callus of *G. asiatica* contain significant amount of flavonoids and immense antioxidant potential which can be correlated to its diverse medicinal values.

Keywords: Folk Medicine, Callus induction, Flavonoids, Quercetin, Antioxidant activity.

INTRODUCTION

Grewia asiatica Mast. (syn. *G. subinaequalis* DC) is a member of family the Tiliaceae that yield edible fruits. The fruit is astringent and stomachic. The unripe phalsa fruit alleviates inflammation and is administered in respiratory, cardiac, and blood disorders, as well as in fever reduction. Furthermore, an infusion of the bark is given as a demulcent, febrifuge, and treatment for diarrhoea [1]. The root bark is employed in treating rheumatism. The leaves are applied on skin eruptions and they are known to have antibiotic action.

The fresh leaves are valued as animal fodder. The bark is used as a soap substitute in Burma. A mucilaginous extract of the bark is useful in clarifying sugar. Fibre extracted from the bark is made into rope. The wood is yellowish-white, fine-grained, strong, and flexible. It is used for archers' bows, spear handles, shingles, and poles for carrying loads on the shoulders. Pruned stems serve as garden poles and for basket-making. Leaves are useful in elephantiasis, inflammations, leprosy, leucoderma, diabetes fever, diarrhoea, gout, rheumatoid arthritis and bronchitis [2]. Ayurveda and other traditional system of medicine support leaves of *G. asiatica* as anti-diabetic, which are efficacious and economical, as compared to synthetic drugs.

The flowers have been found to contain grewinol, a long chain keto-alcohol, tetratricontane 22-ol 13-one [3]. The phalsa seeds produce approximately 5% yield of a bright yellow oil that contains 8% palmitic acid, 11% stearic acid, 13.5% oleic acid, and 64.5% linoleic acid with 3% unsaponifiable [1]. The presence of antihyperglycemic activity in the fruit, stem bark and leaves of *Grewia asiatica* provides a hopeful sign for the presence of some new hypoglycemic agents [4]. Plant parts are found to have all the essential mineral elements, carbohydrates, proteins, fatty acids and other active metabolites like flavonoids, tannins, phenols, alkaloids, steroids and terpenoids etc.. Due to the presence of active metabolites plant is supposed to have high antioxidant activity that may be the reason of high medicinal potential of plant. Medicinal plants typically contain mixtures of different chemical compounds that may act individually or in synergy to improve the health of common man. Antioxidant supplements rich in plants may be used to help the human body in reducing the oxidative damage by free radicals [5].

The present investigation deals with the *in vivo* and *in vitro* plant parts for qualitative analysis of extracts in various solvents, quantitative estimation of total flavonoids, and quantitative estimation of Quercetin and estimation of antioxidant activity.

MATERIAL AND METHOD

The plant *Grewia asiatica* Mast. was collected from the Mahesh Nagar area of Jaipur, Rajasthan and was verified from the specimen number RUBL20950 of Herbarium, Department of Botany, UOR Jaipur.

Callus Induction and Establishment

For callus establishment the fresh and healthy plant twigs were collected and surface sterilized with detergent followed by 0.1% mercuric chloride for 1 min and rinsed with distilled water. Sterilized leaf discs and nodal segments were used as explant for the induction of callus. The explants were inoculated on the semisolid MS media supplemented with 3% sucrose and various concentrations of growth regulators like NAA (0.25mg/lt, 0.5mg/lt, 1.0 mg/lt, 1.5mg/lt and 2.0 mg/lt), 2,4-D (0.5mg/lt, 1.0mg/lt, 1.5mg/lt and 2.0mg/lt) and the combination of NAA and BAP. Agar agar (0.8%) was used as solidifying agent.

The induced callus was then subcultured on MS media with various concentrations of BAP (0.25mg/lt, 0.5mg/lt, 1.0mg/lt, 1.5 mg/lt and 2.0mg/lt) with the aim to stimulate the rate of cell division for enhancement of callus growth.

The developed undifferentiated homogenous cell mass was repeatedly subcultured to maintain cell growth. The collected cell mass was then used for further investigation.

Qualitative Analysis for Flavonoids

The *in vivo* (stem and leaf) and *in vitro* (callus) plant parts were separated and materials collected were allowed to dry at room temperature. The dried plant materials were ground in powdered form and used for various experiments.

Extracts were prepared by the method described by Savithramma *et al.*[6]. 5gms of each plant sample (leaf, stem and callus) was weighed an were soaked in 25ml of ethanol, methanol, hexane, chloroform, petroleum ether, ethyl acetate and distilled water in the ratio 1:5 (weight: volume) These were allowed to stand at ambient room temperature for 48 hours. The soaked plant powder was filtered and used as crude extract. All the extracts were centrifuged at 5000 rpm for 15 mins. The supernatants collected were tested for the presence of Flavonoids. For this purpose following methods were used

(a). 1 ml of each sample was added to 1ml of 1N NaOH. A drop of conc. HCl was added to each mixture and analysed for the reaction [7].

(b). To 2ml of test solution, 0.5ml alcohol was mixed. Then a bit of magnesium and one or two drops of concentrated HCl were added and heated. The mixtures were analyzed for the reaction [8].

Quantitative Estimation of Total Flavonoids

Aluminium chloride colorimetric method was used for flavonoids determination [9]. *In vivo* and *in vitro* plant part extracts (1gm/10ml) prepared in ethanol (0.5 ml) and separately mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminium chloride, 0.1ml of 1 M sodium potassium tartrate and 2.8 ml of distilled water. It was kept at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm. The calibration curve was obtained by preparing Quercetin solutions in range of concentrations 0.1mg to 1.0mg/ml.

Isolation of Quercetin through Thin Layer Chromatography

Thin layer chromatography of plant extracts was performed with the aim to confirm the presence of flavonoid 'Quercetin' in the plant parts. Extraction of flavonoids was done in different fractions [10]. The dried samples were separately soxhlet extracted in 80% methanol (100 ml/gm dry weight) on a water bath for 24 hrs. Each of the extracts was concentrated and reconcentrated in petroleum ether (40°-60°C) (fraction-I), ethyl ether (fraction-II) and ethyl acetate (fraction-III) in succession. Each of the steps was repeated three times to ensure complete extraction in each case. Fraction I was rejected since it was rich in fatty substances whereas fraction II was analysed for the free flavonoids in each of the samples. Fraction III of each of the test samples was hydrolysed by refluxing with 7% H2SO4 (10 ml/gm residue) for 5 hours. The mixture was filtered and the filtrate extracted with ethyl acetate in a separating funnel. The ethyl acetate layer was washed with distilled water till neutrality and dried. The residues were taken up in small volumes of ethanol separately and then subjected to various tests for quercetin.

The glass plates (20 x 20 cm) coated with silica gel 'G' (0.2-0.3 mm thick and 30 gm/60 ml distilled water) were dried at room temperature. The dried plates were activated at 100°C for 30 minutes in an oven and cooled at room temperature. Ethyl ether and ethyl acetate fractions from each samples were separately applied 1 cm above the edge the plates along with standard reference compound (quercetin). These glass plates were developed in a closed chromatography chamber containing about 200 ml of solvent mixture of n-butanol, acetic acid and water (4:1:5, upper layer). The developed plates were air dried and visualized under UV light which showed fluorescent spots. The developed plates were then placed in a chamber saturated with ammonia vapours to observe the colour of spots and plates were also placed in a chamber saturated with Iodine vapours to observe the colour of spots. The developed plates were sprayed with 5% ethanolic ferric chloride solution to observe the colour of the spots (in both the fractions II and III). Rf values were calculated for isolated samples and compared with coinciding standard.

Isolation and Quantitative Estimation of Quercetin through HPLC

HPLC - gradient grade methanol, acetonitrile and Authentic standards Quercetin (RM 6191) were purchased from Himedia Laboratories (Mumbai, India).

The methanolic extract of plant samples were prepared by soxhlet extraction and were evaporated to dryness. The samples were then heated under reflux for 1hour with 6 ml of 25% hydrochloric acid and 20 ml Methanol, the hydrolysate was diluted with methanol to 100ml and filtered. 1ml of this solution was allowed in the injection vial for HPLC analysis. Quercetin in the samples was identified by comparison of their retention times (Rt) with the standard Quercetin.

The HPLC equipment comprised of Agilent 1100/1200 Quaternary Pump (G1311A), Thermostatted Autosampler (G1329A), Diode Array Detector (G1315D) and Automatic Fraction Collector (G1364C). The chromatographic analysis was performed on a 250 mm × 4.6 mm C18 reverse phase column packed with 5µm diameter particles. The mobile phase used was HPLC grade Methanol: Acetonitrile: Water (60:20:20 v/v/v). The mobile phase was filtered through a 0.45 µm membrane filter. Flow rate of mobile phase was kept at 1 ml min^{-1} with pressure 122.6 bar. Injection volume was kept at 5µl. RP-HPLC separation of standard Quercetin at 358 nm. The Chromatographic peaks of the analytes were confirmed by comparing their Retention time and UV Spectra with those of the reference standards. Quantitative analysis of Quercetin was carried out by the integration of the peak using external standard method. All chromatographic operations were carried out at ambient temperature.

Estimation of Antioxidant Activity

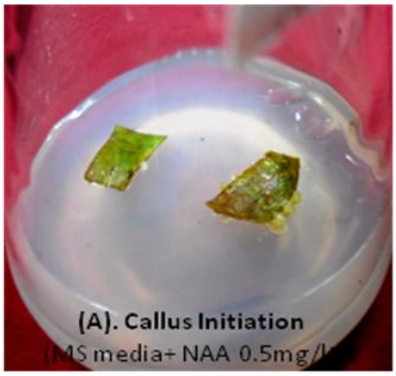
The antioxidant activity of the ethanolic extracts was determined on basis of their scavenging activity of the stable diphenyl-2-picryl hydrazyl (DPPH) free radical. DPPH is stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis. The phenomenon of discoloration of the free radical through neutralization by plant extract was used for estimation of activity [11]. 1ml of each extracts was added to 3 ml of 0.004% ethanolic DPPH free radical solution. After 30 minutes the absorbance of the preparations were taken at 517 nm by a UV spectrophotometer which was compared with the testing preliminary radical scavenging activity of a standard ascorbic acid concentrations (1-500 µg/ml). The results are expressed as IC50 values. The discolouration of sample was presented in terms of percent inhibition of radical scavenging ability that was calculated as

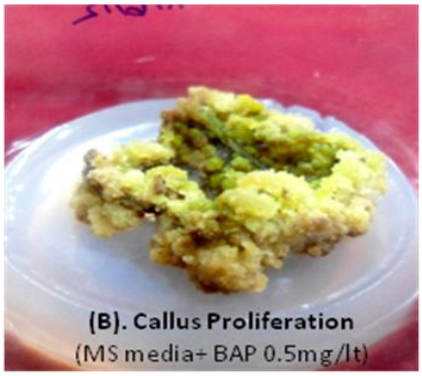
Percent inhibition = [(Abs. of control – Abs. of sample)/ Abs. of control] *100

Ascorbic acid was used as positive control. Percent inhibition of free radical was plotted against the sample concentration in order to calculate the IC50 value. It is defined as the amount of sample necessary to decrease the absorbance of DPPH by 50% that was calculated from the curve.

RESULTS AND DISCUSSION

Callus from leaf discs and nodal explant was best induced on MS media with NAA 0.5mg/lt and was best established on various concentrations (0.5mg/lt and 1.0mg/lt) of BAP.





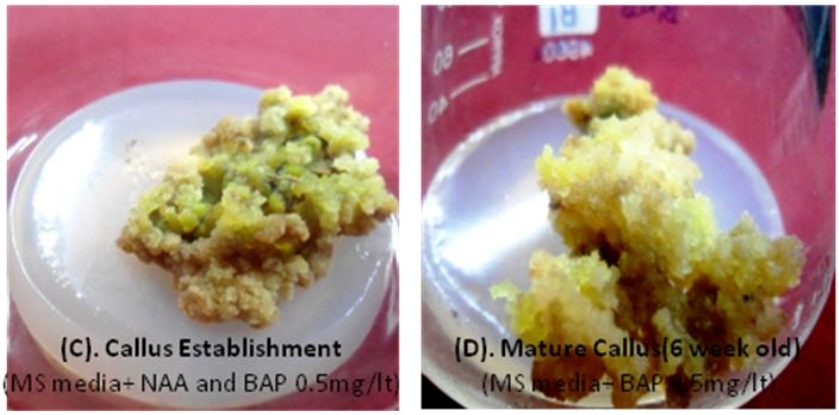


Fig. 1: Various Stages for Callus Induction and establishment for *G. asiatica*

The flavonoid content in the *in vivo* and *in vitro* plant parts was found to be present in the alcoholic, chloroform and aqueous solvents. In the spectrophotometric assay the highest concentration of total flavonoids was present in the ethanolic solvent of leaf (90.67 mg/ gm of dry tissue).

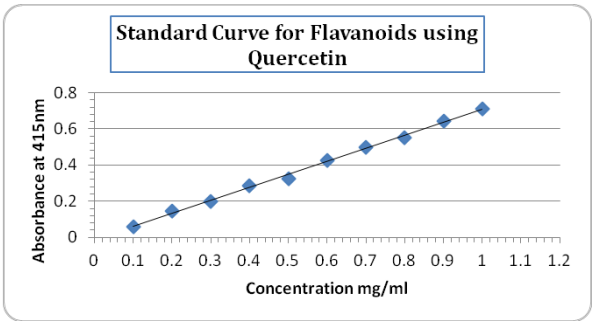


Fig. 2: Reference Curve for Quantification of Flavonoids

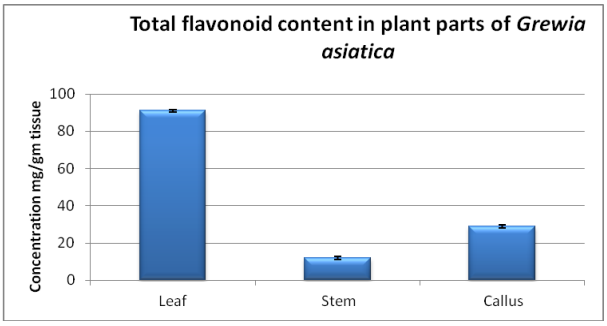


Fig. 3: Quantification of Total Flavonoids in plant parts of *G. asiatica*

Through the Thin layer chromatography Quercetin was found to be present in all plant parts leaf, stem and callus. When the developed plates were sprayed with 5% ethanolic ferric chloride solution it showed spots which coincided with that of the standard reference quercetin (bluish grey) when plates were placed in a chamber saturated with ammonia vapours, it also showed deep yellow colour of quercetin.

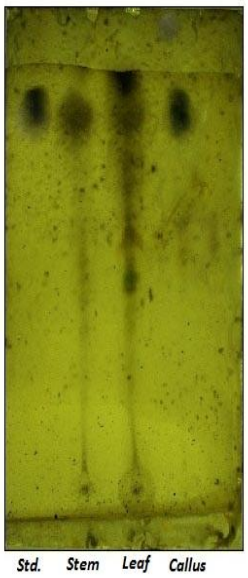


Fig. 4: Thin Layer Chromatogram of *G. asiatica* plant parts against Quercetin Reference

Rf value (0.82) of quercetin isolated from the samples coincided with the Rf value of standard quercetin (0.82). The plates developed under UV light showed fluorescent spots in fraction III coinciding with the standard sample of quercetin (Blue).

When isolated quercetin was subjected to HPLC, it showed retention time 5.915 min in leaf sample and 5.942 min in callus sample, which coincided with that of standard quercetin (6.035 min).

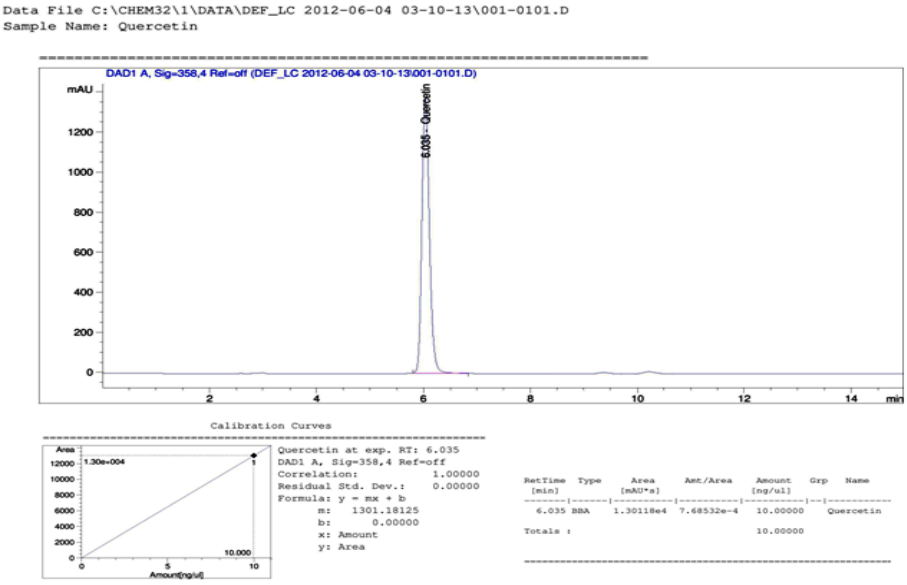


Fig. 5: HPLC Chromatogram of Standard Quercetin

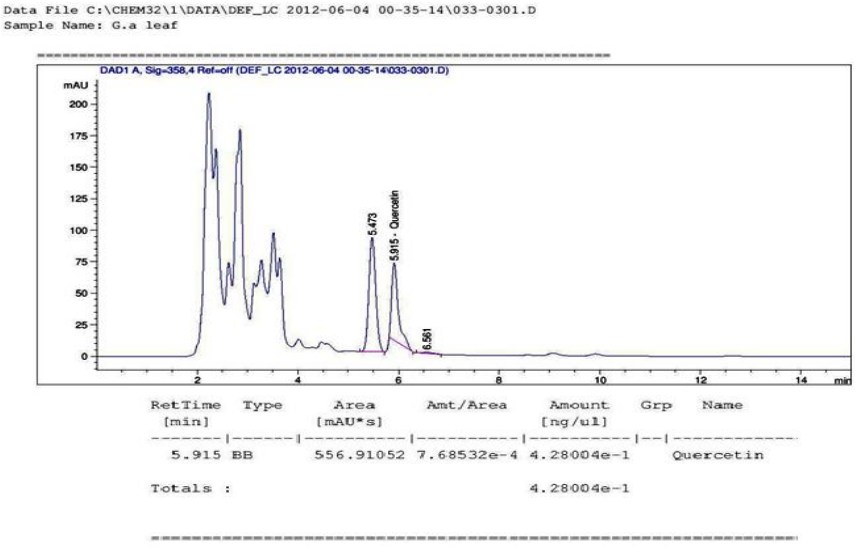


Fig. 6: HPLC Chromatogram of *G. asiatica* leaf

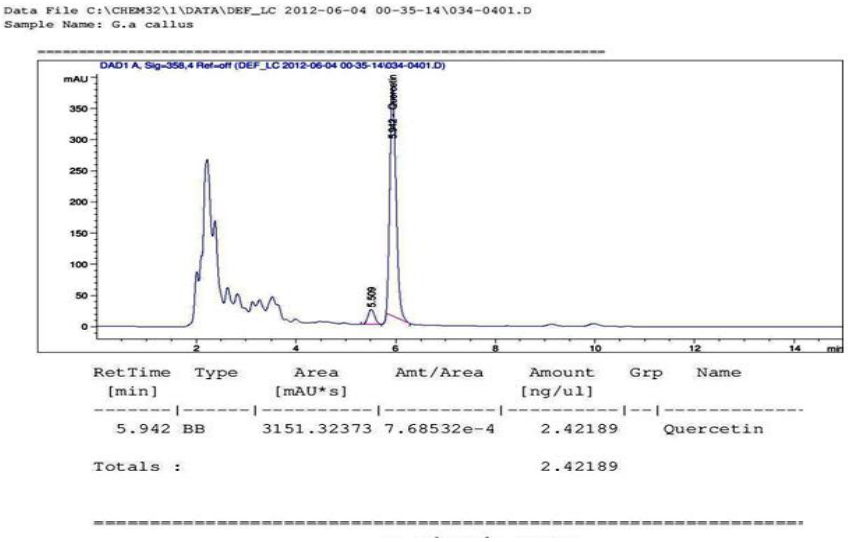


Fig. 7: HPLC Chromatogram of *G. asiatica* Callus

The amount of Quercetin in plant samples was analysed by comparing with the standard curve with known amount of quercetin (10 ng/µl). the amount of Quercetin in the leaf sample was found to be 4.28 ng/µl that was about double that of the callus 2.42 ng/µl.

Antioxidant activities of the fruit and stem extract was found to be higher than leaf and callus by the DPPH assay. *G. asiatica* plant parts were found to have good antioxidant potential when compared to ascorbic acid standard. The IC_{50} value and Antioxidant potential are two inversely related terms. The IC_{50} value for the fruit extract was minimum. Callus showed highest IC_{50} value and least antioxidant potential.

The antioxidant potential of G. asiatica plant parts were found in range from 77% to 98.2 %., while the antioxidant potential of Ascorbic acid was 67.5%. The antioxidant potential of fruit, leaf and stem was found to be higher than the reference standard Ascorbic acid. In other words the *in vivo* plant parts have immense antioxidant potential, therefore have good medicinal values.

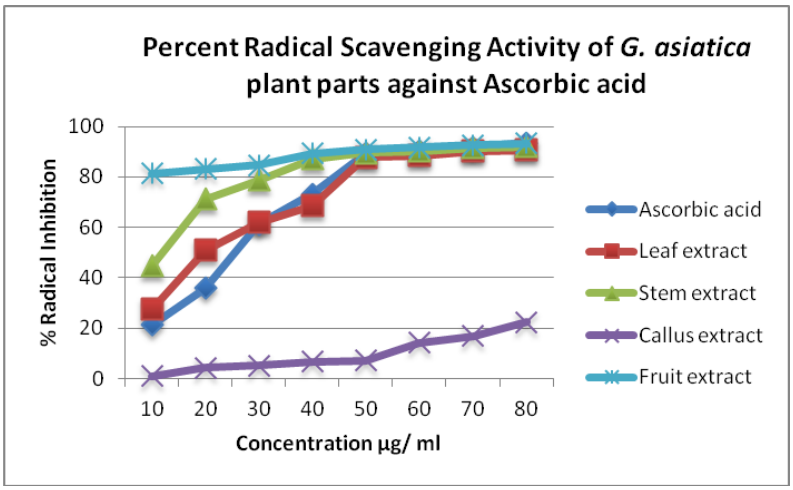


Fig. 8: Antioxidant activity analysis in Plant parts of *G. asiatica*

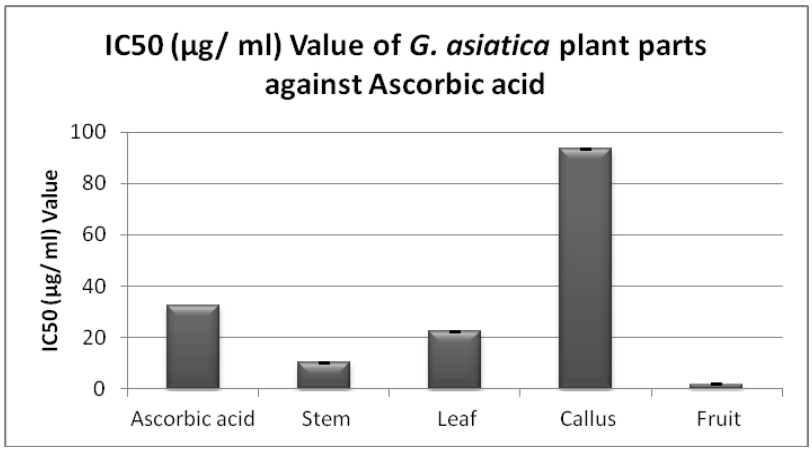


Fig. 9: IC 50 Values for Plant parts of *G. asiatica*

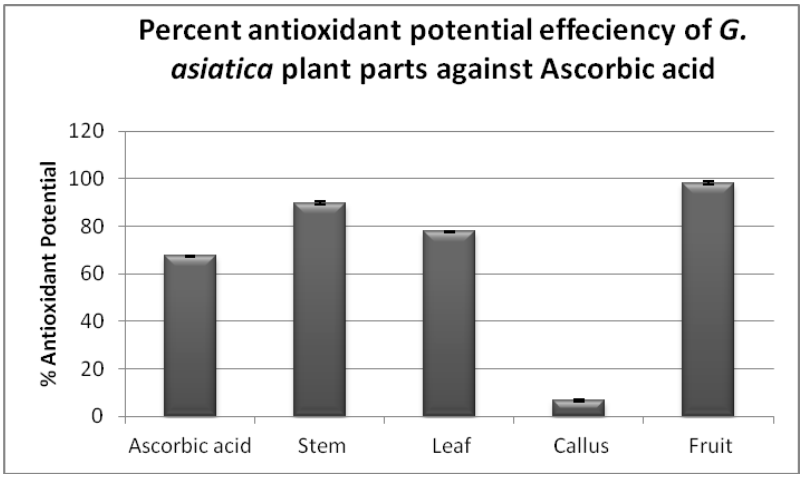


Fig. 10: Antioxidant potential of Plant parts of *G. asiatica*

CONCLUSION

Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenols. Quercetin is the major bioflavonoid in the human diet. These compounds possess abroad spectrum of chemical and biological activities including radical scavenging properties. Its reputation as an antioxidant stems from the reactivity of phenolic compounds with free radical species to form phenoxy radicals which are considerably less reactive. Additionally, one can envision a polyphenolic compound easily oxidizable to a quinoid form (similar to vitamin K) and participating in the redox chemistry of nature. The minimum antioxidant activity of callus of *G.asiatica* can be linked with the presence of minimum amount of flavonoids in it. The present investigation revealed that the leaves, stem and callus of *G. asiatica* contain significant amount of flavonoids and immense antioxidant potential. This study helped to correlate relationship of secondary metabolite flavonoid to possible biological activities and evaluated *G. asiatica* plant as potential sources of natural bioactive chemicals.

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