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Investigation on *in vitro* Antimicrobial and Anti-inflammatory Activity of *Gardenia latifolia* Leaf Extracts in Prospecting New Therapeutics

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Abstract

This research investigates the phytochemical composition and therapeutic potential of *Gardenia latifolia*, a plant species historically recognized for its medicinal properties but lacking contemporary scientific scrutiny. The study focuses on evaluating the phytochemicals present in aqueous and ethanolic extracts of *G. latifolia* leaves and assessing their anti-inflammatory and antimicrobial activities. Through a series of qualitative and quantitative analyses, the study identifies the presence of alkaloids, flavonoids, glycosides, tannins, saponins, and terpenoids in the extracts. Total polyphenolic and flavonoid contents are determined using standard methods, revealing significant concentrations in both extract types. Furthermore, the anti-inflammatory activity of the extracts is assessed through an inhibition of albumin denaturation assay, demonstrating notable inhibition comparable to the standard drug ibuprofen. Additionally, the antimicrobial efficacy against oral and enteric pathogenic bacteria is evaluated using agar well diffusion method, showcasing promising inhibitory effects particularly in the ethanolic extract. These findings underscore the therapeutic potential of *G. latifolia* extracts, highlighting their anti-inflammatory and antimicrobial properties, and suggest avenues for further pharmacological investigations to exploit their therapeutic benefits with minimal side effects.

Key Words: Phytochemicals, *Gardenia latifolia*, Antimicrobial, Anti-inflammatory

Introduction

Medicinal plants are the reservoirs of potentially useful chemical compounds which could serve as newer leads and clues for modern drug design (Deodhar, and Shende, 2016). Correlation between the phytoconstituents and the bioactivity of plant is desirable to know for the synthesis of compounds with specific activities to treat various health ailments and chronic diseases as well (Pandey, *et al*., 2013). The phytochemical research based on ethno-pharmacological information is generally considered an effective approach in the discovery of new anti-infective agents from higher plants (Duraipandiyan, *et al*., 2006). Historically pharmacological screening of compounds of natural or synthetic origin has been the source of innumerable therapeutic agents. Random screening as tool in discovering new biologically active molecules has been most productive in the area of antibiotics (Minhas, *et al*., 2013).

Gardenia latifolia (Rubiaceae) is commonly known as Indian boxwood or Ceylon boxwood, is a densely foliaceous small tree that occurs throughout the greater parts of Indian common in deciduous forests along the streams (Tamilselvi, *et al*., 2017). The plant is deciduous tree, up to 5m high; branches woody, terete, stout. Leaves opposite, stipulate, subsessile to petiolate; petioles 2-6mm long, flattened, glabrous; stipules 0.5-1.5 × 0.5-1.5 cm, inflated, connate, truncate or slightly toothed above, membranous; lamina ovate, orbicular, entire, apex broadly acuminate, coriaceous, glabrous above, pubescent below specially on veins. Inflorescence terminal, solitary or 2-together. Flowers white to yellowish, pedicellate, fragrant; pedicels 0.6-0.8 cm long, smooth, glabrous. Fruits globose, 3-5 × 2-3.5 cm, woody, with stout beak (Ray and Rahaman, 2018). The stem bark and fruits are reported to be used in the treatment of various ailments such as snake bite, skin diseases, stomach pais, cariesin humans and ephemeral fever in live stocks (Reddy, *et al*., 2006; Madava Chetty *et al*., 2008; Dr. Duke's) Fruits are used for making perfumes (Chandra Prakash, K.2009).

But in present time very little of say almost no work is available in context to investigation on *G. latifolia* plant in order to generate any scientific data regarding the therapeutic significance of this plant species. Thus, the present investigation was intended to evaluate the phytochemicals in aqueous and ethanolic extracts of *G. latifolia* leaves and to investigate its anti-inflammatory activity and antimicrobial activity *in vitro* against oral and enteric pathogenic bacteria to generate latest scientific data.

Materials and Methods

Sample Collection

The plant material specifically the leaves of *Gardenia latifolia* were collected from forest region of archeological site *Bhimbetka* rock shelters of central India, district Raisen,

near, Bhopal (M.P) India and authenticated with the help of botanist and literature at Molmet Biotech Research Pvt. Ltd., Bhopal, India.

Extraction of phytochemicals

The washed and cleaned leaves of *G.latifolia* were allowed to dry in shade for 2 weeks and then grounded into fine powder in mixer grinder followed by defatting in petroleum ether overnight. Aqueous and ethnanolic extracts were prepared by soxhlation method 200 ml of pure distilled water and 50% ethanol separately as solvents of extraction where 15 grams of dried leaf powder was subjected to soxhlet extraction in each setup. The temperature of the heating mantel for Soxhelation was maintained at 70^{o}C and 85^{o}C for aqueous and ethanolic extraction respectively which carried out till the exhaustion of the merc is achieved (Handa, 2008; Tenguria, *et al*., 2012). The extract was concentrated in water bath and subjected to phytochemical analysis both qualitatively and quantitatively.

Preliminary Phytochemical Analysis

For preliminary testing and other *in vitro* pharmacological studies a stock solution of 1 mg per ml in sterile distilled water was prepared with dried concentrated extract of *G.latifolia* leaves that were subjected to the phytochemical test using Harbourne's (1983) methods. Using about 100 µl of stock was diluted with distilled water tests conducted in preliminary analysis were alkaloid, tannins, terpernoids, saponins, flavonoids and glycosides by Dragendorff's test, ferric chloride test, chloroform-H_2SO_4 ring test, froth test, lead acetate test and Benedict's test respectively.

Estimation of Total Polyphenolic Content

The total phenolic content of the extracts was determined using to the Folin-Ciocalteu method (Singleton *et al*., 1999) with suitable modification. The extracts were suitably diluted with their respective solvents and oxidized with Folin-Ciocalteu reagent, and the reaction was neutralized with sodium carbonate. After 60 minutes of incubation in dark, the absorbance of the resulting blue colour was measured at 650 nm in spectrophotometer (Electronic India model EI-2305). Using Gallic acid (Thomas Baker Chemicals Pvt. Ltd. India) as standard total phenolic content (standard curve was prepared using concentrations 0.125-2 mg/L) was expressed as mg GA equivalent/L of extract.

Estimation of Total Flavonoid Content

The estimation of total flavonoid content in plant extracts was done by aluminum chloride complexation in accordance with Miliauskas, *et al*., (2004) and Marinova, *et al*., (2005) with reference to the literatures with some modification suitable for present experimental conditions (Chandra, *et al*., 2014). The extracts were suitably diluted with their

respective solvents and mixed with 2% aluminum chloride solution and incubated at room temperature for 60 minutes. The absorbance of yellow colour developed was measure at 420 nm in spectrophotometer (Electronic India model EI-2305). Using concentration vs absorbance standard curve plot of Quercetin (HiMedia laboratories Pvt Ltd India) as standard flavonoid (prepared from 0.625 to 10 mg/ml concentrations) TFC was expressed as mg quercetin equivalent flavonoid in extracts.

Estimation of *in vitro* Anti-inflammatory Activity

The anti-inflammatory activity of samples will be studied by using inhibition of albumin denaturation according technique described by Mizushima and Kobayashi (1968) and Sakat *et al*., (2010) with suitable modifications. The reaction mixture will consist of test sample or extracts and 1% aqueous solution of bovine albumin fraction, maintaining the neutral pH of the reaction mixture. This is followed byincubation at 37ºC for 20 min and then heated to 50º C for 20 min, after cooling the samples; the turbidity was measured at 660nm.The experiment was performed in triplicate. The Percentage inhibition of protein denaturation was calculated according to Gunathilake, *et al*., (2018) as follows:

$$\text{Percentage inhibition} = \frac{1 - \text{Abs of Sample}}{\text{Abs of Control}} \times 100$$

The decrease in absorbance with increase in drug concentration indicates the increase in anti-inflammatory activity which is compared with the standard drug like ibuprofen.

Antimicrobial Activity

The antimicrobial activity of extracted phytochemical extracts was performed by agar well diffusion method upto 5 dilution of 100 mg per ml stock extract was passed through 0.22 micro size dissociable syringe filter (Moxcare) using a sterile syringe to assure the sterility of extracts. The MTCC bacterial cultures procured from IMTech Chandigarh *Streptococcus mutans* (MTCC-497), *Enterococcus faecalis* (MTCC-439), *Escherichia coli* (MTCC-1687) and *Salmonella enterica* (MTCC-3858) were revived in sterile nutrient broth and prepared upto 0.5 McFarland standards. Using sterile swabspathogenic microbial strain was separately inoculated all over on Nutrient Agar media plates. After this, 5 wells of approx. 6 mm diameter were aseptically punched. Each well was filled with 20 µl of different concentrations of extract. The plates were than incubated for 24 hour at 37^{o}C, thereafter zone of inhibition (in mm) were observed and measured with the help of zone scale.

Results and Discussion

Phytochemical Groups in *G.latifolia* Extracts

The percentage yield & organoleptic properties of hydroethanolic extract *Gardenia latifolia* leaves are mentioned table 1 while the observation of its preliminary phytochemical tests are mentioned in table 2. The assumed quantity of phytochemicals groups or intensity of test reaction was counted on 5+.

Table 1: Organoleptic properties of phytochemical extracts of *Gardenia latifolia*

S.N.	Variables/Quality	Properties	
		Aqueous Extract	**Ethanolic Extract**
1.	% Yield	86%	14.4%
2.	Colour	Greenish	Greenish Black
3.	Texture	Sticky Past	Sticky Past
4.	Smell	Fresh Organic/Bitter	Fresh Organic/Bitter

Table 2: Results of preliminary phytochemical analysis of *Gardenia latifolia* leaf extracts

S.N.	Constituents Tested	Aqueous Extract	Ethanolic Extract
1.	Alkaloids	+4	+4
2.	Flavonoids	+3	+4
3.	Glycosides	+4	+2
4.	Tannins	+4	+3
5.	Saponins	+2	+4
6.	Terpenoids	+2	+2

[(+) means present, & (-) means absent]

According to the results of phytochemical test depicted in table 2, the aqueous and hydroethanolic *i.e.,* ethanolic extract of leaves of *G. latifolia,* is rich in variety of phytochemicals where based on the intensity of chemical reaction, the presence of alkaloids, glycosides and tannins seems to be prominently present in aqueous extracts while flavonoids were in moderately incident and those of saponins and terpenoids were just enough. Whereas, the ethanolic extract were reported to content rich amount of alkaloids, flavonoids and saponins based on the intensity of phytochemical test reactions while tannins were moderately present although glycoside and terpenoids were reported to show limited present in ethanolic extract in present experimentation. The presence of phytochemicals is generally responsible for the biological activity of plant extracts that could useful in development of

new therapeutics and medications upon extensive investigation (Tenguria *et al*., 2013, Alawa *et al*., 2018).

Total Polyphenol and Flavonoidal Content

The result of total polyphenolic content estimation in *Gardenia latifolia* leaf extracts are mentioned in table 3 as mg/ml gallic acid equivalent in extract solution using the equation based on the calibration curve:

$$\mathbf{Y = 0.9495X + 0.0002,\ R^2 = 0.9994}$$

Where: X = absorbance and Y = Gallic acid equivalent (GA).

While the outcomes total flavonoidal content in the extracts was measured using standardcalibration curveplot of standard flavonoid quercetin as mentioned in table 4 with following equation:

$$\mathbf{Y = 0.1292X - 0.0036,\ R^2 = 0.9984}$$

Where: X = absorbance and Y = Quercetin equivalent (QE).

Table 3: Gallic acid as standard concentration vs absorbance at 650 nm to plot standard curve for estimation of phenolics in samples Using Folin-Coeucaltue's Method.

S.N.	GA Concentration in mg/ml	Absorbance at 650 nm
1.	2	1.891
2.	1	0.976
3.	0.5	0.457
4.	0.25	0.228
5.	0.125	0.128

Instrument Used: Single beam visible range digital microprocessed spectrophotometer from Electronic India model EI-2305.

Table 4: Quercetin as standard concentration vs absorbance at 420 nm to plot standard curve for estimation in samples Using $AlCl_3$ precipitation Method.

S.N.	Quercetin Concentration in mg/ml	Absorbance at 420 nm
1.	10	1.280
2.	5	0.671
3.	2.5	0.296
4.	1.25	0.151
5.	0.625	0.087

Instrument Used: Single beam visible range digital microprocessed spectrophotometer from Electronic India model EI-2305.

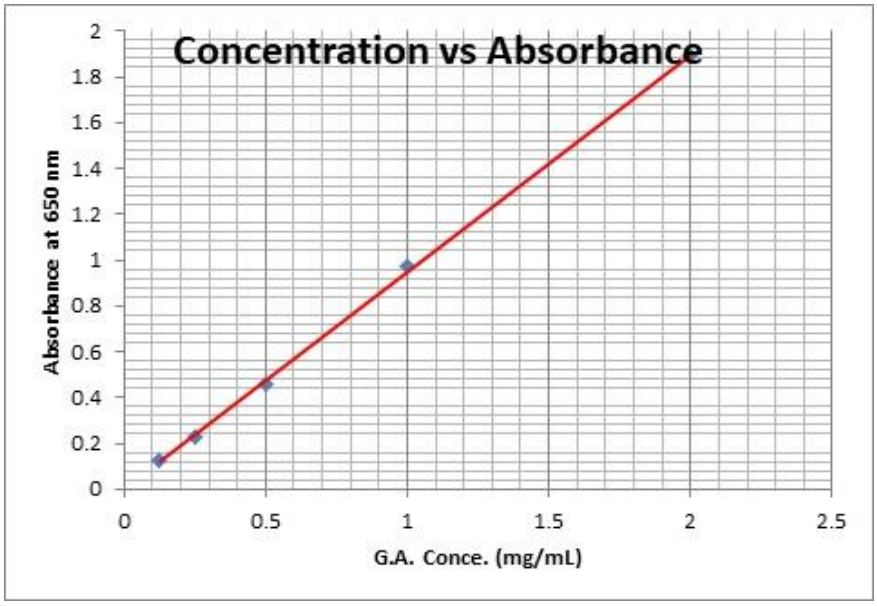

Figure 1: *Standard Plot for known concentration of Gallic acid Standardat 650 nm. The Graph is obtained from Excel 2013 linear regression function*

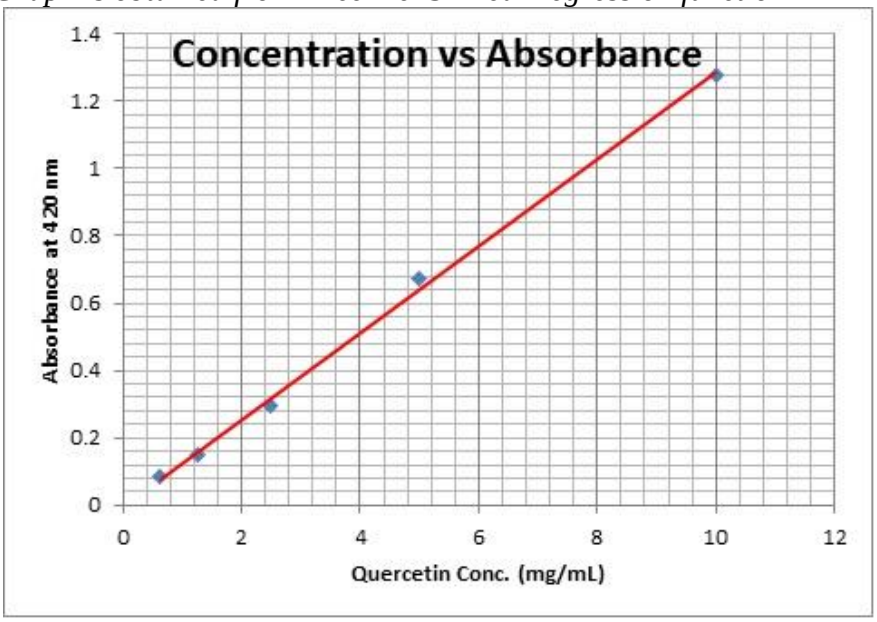

Figure 2: *Standard Plot for known concentration of Quercetin Standard. The Graph is obtained from Excel 2013 linear regression function.*

The graphs of calibration curve of standard Gallic acid and Quercetin are depicted in figure 1 and figure 2 generated on Excel 2013 linear regression and trend function. According to which the total polyphenolic content in aqueous and ethanolic extract was reported to be 52.9% and 31.2% GAE respectively, while the total flavonoid in both aqueous and ethanolic extract was estimated to be 34.04% and 29.86% QE respectively (See Table 5).

Table 5: Estimation of total phenolic and flavonoid content in *Gardenia latifolia* leaf extracts in present study.

S.N.	Sample Extract	GAE TPC	QE TFC
1.	*G.latifolia* leaf Aqueous Extract	52.9%	34.04%
2.	*G.latifolia* leaf Ethanolic Extract	31.2%	29.86%

The phenolic content of any plants is directly related to their antioxidant properties. Phenolic compounds act as reducing agents, hydrogen donors, and are capable of scavenging free radicals (Wojdylo, *et al*., 2007). Presence of considerably good amount of phenolics in aqueous and hydroalcoholic leaf extracts of *G.latifolia* may contribute significantly to the antioxidant properties. Because of these properties, this plant might have been used in several traditional herbal medications. The antioxidant response of phenolic compounds varies remarkably, depending on their chemical structure (Gracia, *et al*., 1997). In addition, there may be some interference rising from other chemical components present in the extract, such as sugars or ascorbic acid (Singleton and Rossi, 1965). Flavonoids are the important polyphenols of plant which are responsible of many pharmacological properties of plant extracts. The hydrogen donating property of the polyphenolic compounds is responsible for the inhibition of free radical induced lipid peroxidation and thus phenols of plant extracts are highly effective antioxidants and explained with respect to their total phenolic and flavonoid contents, in good correlation (Yen *et al*., 1993; Tenguria *et al*., 2013). Higher the amount of polyphenols in plant extracts indicates the higher potential of antioxidant, antimicrobial, anti-cancer and other pharmacological activity (Tenguria *et al*., 2012; Reddy, *et al*., 2021).

Assessment of *in vitro* Anti-inflammatory Activity

In the present study, the protein denaturation bioassay was performed for *in vitro* assessment of anti-inflammatory property of aqueous and ethanolic extract of *G.latifolia* leaves compared to the activity of NSAID Ibuprofen are mentioned in table 6 in terms of percentage inhibition on comparative basis. From the results as depicted in table 6, the aqueous extract showing a percentage inhibition of albumin protein denaturation by 91.2% which is substantially comparable to the denaturation inhibition potential of NSAID Ibuprofen which was 98.8%; however the ethanolic extract of *G.latifolia* is also showing protein denaturation inhibition activity in sufficient amount as 87.4% but it is lesser compared to the aqueous extract. This indicates *G.latifolia* phytoconstituents have anti-inflammatory potential.

Table 6: Anti-inflammatory activity of *Gardenia latifolia* leaf extract and Ibuprofen by percentage inhibition in protein denaturation activity

S.N.	Drug/Sample Extract Used	Concentration	Percentage Inhibition
1.	*G.latifolia* leaf Aqueous Extract	10 mg/ml	91.2%
2.	*G.latifolia* leaf Ethanolic Extract	10 mg/ml	87.4%
3.	NSAID Ibuprofen.	10 mg/ml	98.8%

Inflammation is a substantial reaction to damage, disease or destruction portrayed by heat, redness, pain, swelling and disturbed physiological functions (Dharmadeva, *et al*., 2018). Denaturation of protein has an unpredictable mechanism which includes modification in electrostatic hydrogen, hydrophobic and disulfide bonding (Sen, *et al*., 2015). Most biological proteins lose their biological function when denatured. Denaturation of proteins is a well-documented cause of inflammation (Leelaprakash, and Dass, 2011). Hence, by inhibition of protein denaturation, inflammatory activity can be inhibited (Sangeetha and Vidhya, 2016).

Assessment Antimicrobial Properties

The antimicrobial activity of aqueous and hydroethanolic extract of *G.latifolia* leaves was evaluated against two oral and two enteric pathogenic bacterial species whose observed inhibitory response is mentioned in table 7 and table 8 respectively as zone of inhibition. It is clear that the ethanolic extract was showed more efficient inhibition of test bacterial species compared to aqueous extracts.

The undiluted aqueous extracts showed a maximum zone of inhibition of 9 to 10 mm against the test bacteria while at lowest concentration of 6.25 mg/ml there was no inhibition observed against any bacterial species. The *S.mutans* and *S. enterica* were inhibited upto concentration as low as 12.5 mg/ml with zone of inhibition sizes 7 and 8 mm respectively while *E.coli* and *E.faecalis* were inhibited upto 25 mg/ml with 8 mm sized zone of inhibition in both. It is obvious that the undiluted ethanolic extract of *G. latifolia* leaves showed maximum zone of inhibitions against all test bacterial, however *E.coli* was maximally inhibited with zone of inhibition as 21 mm followed by *S. enterica*, *S. mutans*, and *E. faecalis* with 20 and 19 mm sized zone of inhibitions. The inhibitory potential of extracts gradually decreases with decrease in extract concentration, where the least inhibition observes at 6.25 mg/ml extract concentration with 10 mm of zone size for all the test bacterial species.

Table 4: Results of antimicrobial sensitivity test due to aqueous extract of *Gardenia latifolia* leaves against test bacterial strains

S.N.	Phyto Extract Concentration in mg/ml	Zone of Inhibition (in mm) against			
		S. mutans **(MTCC-497)**	***E. faecalis*** **(MTCC-439)**	***E. coli*** **(MTCC-1687)**	***S. enterica*** **(MTCC-3858)**
1.	**100**	10 mm	09 mm	10 mm	10 mm
2.	**50**	08 mm	08 mm	08 mm	09 mm
3.	**25**	08 mm	08 mm	08 mm	09 mm
4.	**12.5**	07 mm	0	0	08 mm
5.	**6.25**	0	0	0	0

Table 4: Results of antimicrobial sensitivity test due to hydroethanolic extract of *Gardenia latifolia* leaves against test bacterial strains

S.N.	Phyto Extract Concentration in mg/ml	Zone of Inhibition (in mm) against			
		S. mutans **(MTCC-497)**	***E. faecalis*** **(MTCC-439)**	***E. coli*** **(MTCC-1687)**	***S. enterica*** **(MTCC-3858)**
1.	**100**	19 mm	19 mm	21 mm	20 mm
2.	**50**	16 mm	17 mm	17 mm	15 mm
3.	**25**	15 mm	13 mm	16 mm	15 mm
4.	**12.5**	11 mm	11 mm	12 mm	11 mm
5.	**6.25**	10 mm	10 mm	10 mm	10 mm

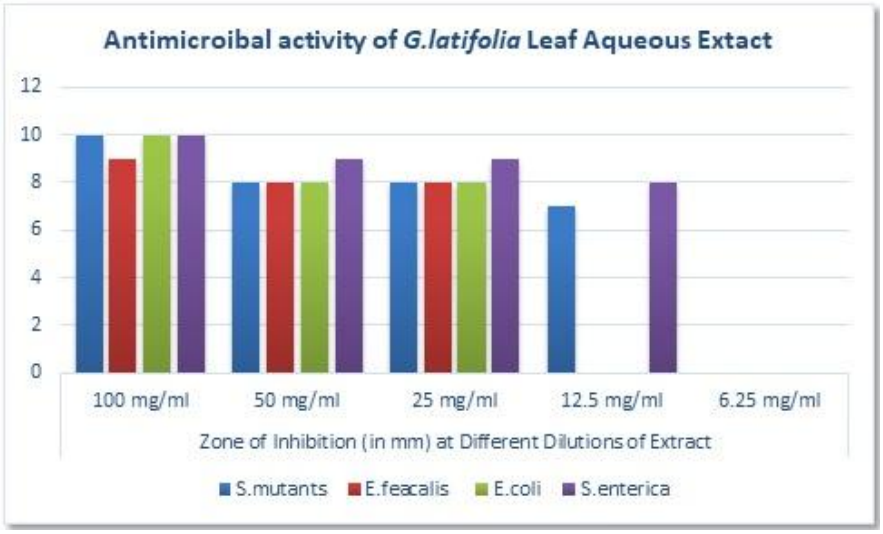

Figure 3: *Graphical representation of the efficacy of Aqueous Extract of G. latifolia* leaves *against the test oral and enteric pathogenic bacteria*

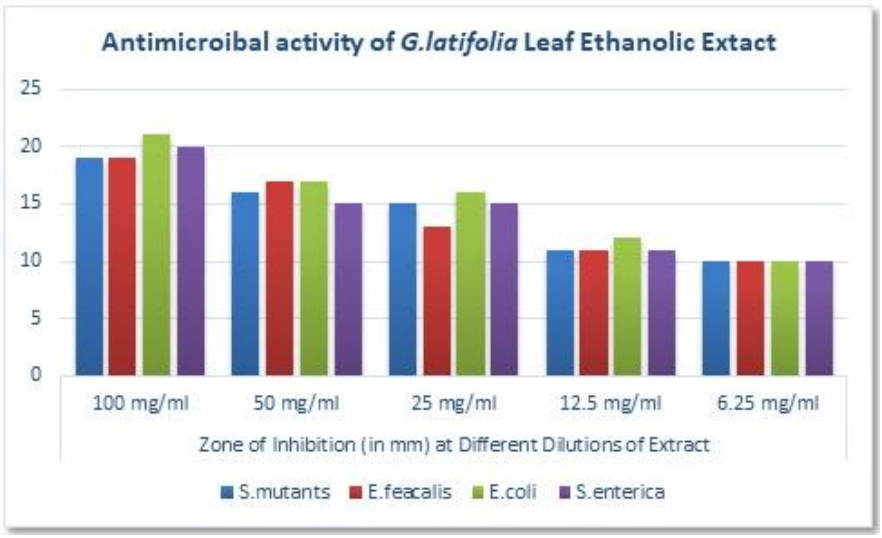

Figure 4: *Graphical representation of the efficacy of Ethanolic Extract of G. latifolia* leaves *against the test oral and enteric pathogenic bacteria*

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the *in vitro* antibacterial activity assay (Tona *et al.,* 1998). Many reports are available on the antiviral, antibacterial, antifungal, anthelmintic, antimolluscal and anti-inflammatory properties of plants (Govindarajan *et al.,* 2006). Present experiment suggests the inhibitory spectrum of aqueous and ethanolic extracts of *G.latifolia* leaves were substantial in terms of anti-inflammatory and antimicrobial under *in vitro* assays, however, further investigations are needed to be done on number of pharmacological parameters so that therapeutic significance of phytochemicals from this plant taken under present study could be elucidated.

Conclusions

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The aqueous and ethanolic extracts from the leaves of *Gardenia latifolia* are reported to be rich in various phytoconstituents with special reference to polyphenols and flavonoids which are mostly responsible for biological and pharmacological properties of any plant. The extracts in present study showed substantial amount of *in vitro* anti-inflammatory and antimicrobial activities against test bacterial strains encourages possibilities in development of new therapeutics and drugs upon further extensive investigation on this plant with less or no side effects in this modern scenario.

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