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Estimation of total phenolic and flavonoid contents and evaluation of antioxidant activity of different parts of *Cassia angustifolia Vahl*

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Abstract
The Caesalpiniaceae family includes the traditional medicinal plant *Cassia angustifolia* Vahl. The species is credited with a number of bioactive components, including phenolics, flavonoids, glycosides, coumarins, saponins, terpenoids, and alkaloids, among others, that contribute to a wide spectrum of therapeutic characteristics. The goal of the current study was to investigate the effect of different climatic conditions on total phenolic and flavonoid contents as well as on the antioxidant potential of different parts (root, stem, and leaves) of *C. angustifolia.* The plants were collected from three different states of India-Rajasthan, Gujrat, and Tamilnadu. Results of the current study showed that the selected plant parts possess significant amounts of total phenols and flavonoids. Our research revealed that methanolic extracts of leaves, roots, and stems from Tamilnadu had the maximum antioxidant activity, followed by Rajasthan and Gujarat. Rajasthan also had the highest levels of phenolic and flavonoid content, which was then followed by Tamilnadu and Gujarat. The results of a one-way ANOVA analysis showed that total phenolic and total flavonoid contents as well as antioxidant potential of the selected plant parts from the various sites were not significantly different. As a result, these plant parts can be utilised in place of harmful synthetic antioxidants and can be employed as natural antioxidants like red wine, green tea, and chocolates etc.

Keywords: antioxidant potential, total flavonoid content, total phenolic content, climatic conditions etc

Introduction

As sources of remedies, medicinal plants are frequently employed as complementary therapeutic instruments for the prevention or treatment of numerous ailments. Recent studies have looked into how phenolic components including flavonoids, phenolic acids, tannins, etc. are primarily responsible for the antioxidant activity of plant parts (Nagavani and Rao, 2010; Cartea *et al*., 2010; Kaur and Mondal, 2014) [23, 6, 16]. Phenolics are ubiquitous secondary metabolites in plants. They include about 8000 bioactive chemicals. Numerous biochemical activities, including anti-oxidant, anti-mutagenic, and anti-cancerous, are exhibited by phenols. They can also alter gene expression significantly (Nakamura *et al*., 2003) [25]. More than 4000 flavonoids of a plant origin have been discovered thus far. High flavonoid consumption lowers the risk of cancer and cardiovascular disease (Tapiero *et al*., 2002; Pamulaparthi1 *et al.*, 2016; Ahmed *et al.*, 2016) [33, 1]. Flavonoids are a broad range of secondary metabolites in plants that are polyphenolic phytochemicals with variable phenolic structures. They include flavones, flavanone, flavanols, flavonols, and flavanonols (Hamamatsu *et al*., 2004; Santos *et al*., 2011; Chua *et al*., 2011; Laghari *et al*., 2010) [13,26,9,18]. Vegetables, fruits, flowers, grains, barks, roots, and stems all contain these phytochemicals. According to Cushnie and Lamb (2005) [10], flavonoids show important biological activities such as antibacterial, antioxidant, anti-cancer, and anti-inflammatory activity (Laghari *et al*., 2011) [19].

For all living beings, to survive on this planet, oxygen is a must. About 5% of oxygen is univalently reduced into oxygen-derived free radicals (ROS) like superoxide anions (O^{2-}), hydroxyl (-OH), and nitric oxide (NO), which damage cellular components and cause tissue injury through covalent binding, during the process of oxygen utilisation in a normal physiological and catabolic process (Katsube *et al.*, 2006; Krishna *et al.*, 2019) [15, 17]. Pathological illnesses like ischemia, asthma, arthritis, inflammation, neurodegeneration, Parkinson's disease, mongolism, the ageing process, and maybe dementia can caused due to accumulation of free radicals. When antioxidant capacity is exceeded in biological system, the production of reactive oxygen species (ROS) during metabolism and other processes is increased which result into oxidative stress (Zima *et al*., 2001) [35]. Most frequent free radicals formed from nitrogen include nitric oxide (NO*), peroxy nitrite anion (ONOO*), hydroxy radical, hydrogen radical, and super oxide anion (O2*) (Pamulaparthi *et al.*, 2016; Nagendrappa, 2005) [24]. Antioxidants are compounds which are present in low concentrations as compared to oxidizable substances but they can delay or stop those molecules from oxidising. By interacting with free radicals and functioning as oxygen scavengers, foods rich in antioxidants reduce the risk of chronic diseases such neurodegenerative disorders, cardiovascular diseases, the ageing process, cataracts, cancer, brain dysfunction, and other age-related degenerative diseases. Many synthetic antioxidants, including butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA), and propyl gallate (PG), are used to slow down the oxidation process in order to meet the rising demand for antioxidants, but their safety has long been questioned due to their unfavourable side effects and potential health risks (Pamulaparthi1 *et al*., 2016; Ahmed *et al.*, 2016; Alhawari *et al*., 2021) [1]. Many research studies have turned to natural antioxidants in an effort to locate sources of antioxidants that would be secure, efficient, and affordable (Mundhe *et al*., 2011; Krishna *et al.*, 2019) [22, 17]. Free radical scavengers found in herbal medicines are renowned for their medicinal properties (Hakiman and Maziah, 2009) [12]. To stop the oxidation of the vulnerable substrate, plants create a highly astonishing range of

antioxidant substances, such as carotenoids, flavonoids, ascorbic acid, etc. (Kaur and Mondal, 2014) [16]. Thus, there is a need to research and create natural antioxidants that are more effective and have lesser negative effects (Dela Torre *et al.,* 2017) [11].

The Caesalpiniaceae family includes the traditional medicine plant *C. angustifolia* Vahl. It is sometimes referred to as *Cassia senna* or *Senna makkai*. *C. angustifolia* is a native of Yemen, Saudi Arabia, and Egypt. It is a rapidly growing shrub between 5 and 8 metres tall that is widely farmed in Pakistan and India's hot, dry regions for its fruit and leaves (Laghari *et al.,* 2011) [19]. In both the British and American pharmacopoeias, this plant is included (Bameri *et al.,* 2013) [4]. As an anti-helmenthic, the leaves and pods of *C. angustifolia* are used as a powdered decoction treating intestinal worms. Additionally, it is frequently used as an anti-pyretic for cholera, splenic enlargements, typhoid, anaemia, toxicity, and genotoxicity brought on by *Escherichia coli* (Laghari *et al.,* 2011) [19]. The studies published in the literature and the widespread use of *C. angustifolia* as a folk remedy for a variety of diseases call for more investigation to identify the substances causing its bioactivities (Ahmed *et al.,* 2016) [1]. It has long been used as a purgative in folk medicine and is a well-known medication in traditional medicine (Wu *et al.,* 2009) [34]. The majority of the world's pharmacopoeias also list this plant (Siddique *et al.,* 2010) [29]. It is valued as a medicine because of its cathartic effects and is especially helpful in chronic constipation. Traditional purgatives include the leaves and pods of *C. angustifolia*; the most significant purgative components are Sennosides A, B, C, D, emodin, isorhamnetin, and essential oil (Siddique and Anis, 2007) [28]. *C. angustifolia* is a plant that greatly contributes to the production of commercial medications and has been researched in numerous regions of the world for a variety of therapeutic preparations in various methods. Its leaves are also used as a safe laxative (Arya, 2003; Laghari *et al.,* 2011) [3, 19].

There are very few studies on *C. angustifolia's in vitro* antioxidant tests, despite the fact that many reports have been published on the species' other therapeutic characteristics. Hence in the present study, antioxidant activity along with total phenolic and flavonoid contents was evaluated in different parts (stem, root and leaves) of *Cassia angustifolia vahl* and effect of climatic conditions were also observed on those parameters.

Materials and methods

Sample collection and processing

Different plant parts of *Cassia angustifolia* vahl (stem, root, and leaves) were collecetd from Rajasthan, Gujarat, and Tamilnadu in India. After washing the samples, the various plant sections were separated. Those underwent a 10-day period of room-temperature shade drying. The dry components were crushed into a powder. The powered samples were retained and stored separately in an airtight container for use in additional phytochemical analysis.

Sample extraction

Using methanol as a solvent, the dried plant components were extracted using the cold percolation method. 10 g of the dry powder was placed in a conical flask with 100 ml of methanol and shaken at 120 rpm for 24 hours in an orbital shaker. The extracts were evaporated under vacuum after 24 hours and then filtered using Whatman filter paper no. 1 to remove debris.

Determination of total phenolic contents in the plant extracts

The Folin-Ciocalteau method was used to determine TPC (the total phenolic content) (Singleton *et al.,* 1965; McDonald *et al.,* 2001) [21]. The reaction mixture was made by combining 0.5 ml of methanolic extract solution (1 mg/ml), 2.5 ml of water-dissolved 10% Folin-reagent, Ciocalteu's and 2.5 ml of 7.5% NaHCO3. The mixture was let to stand at 45° C for 15 minutes, and the phenols were then identified using a spectrophotometric technique. At 765 nm of wavelength, the absorbance was measured. The samples were made in triplicate, and the mean value of absorbance was calculated. Methanol was used to prepare the blank simultaneously instead of the extract solution. Gallic acid in methanol standard solution, 100–1000 mg/L, was used to create the standard curve. The amount of gallic acid equivalent (mg of GAE/g of dry weight), a frequent benchmark compound, was used to express the total phenolic content.

Determination of total flavonoid concentrations in the plant extracts

Using a slightly modified version of the aluminium chloride spectrophotometric technique (Chang *et al.,* 2002**)** [8]**,** the concentration of TFC (total flavonoid content) was measured. Plant extracts (0.5 ml) were dissolved in 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water before being incubated at room temperature for 30 minutes. At 415 nm, the reaction mixture's absorbance was gauged. The mean value of absorbance value was calculated for each experiment in triplicate, and values were represented as mean standard deviation. The standard quercetin solution in methanol was used to create the standard curve. The extracts' overall flavonoid concentration was measured in milligram of quercetin equivalents per gram of dry weight.

Extraction of flavonoids

Following the established method of Subramanian and Nagarajan, flavonoids were extracted from various plant parts (1969). Soxhlet extraction was performed on 100 grams of finely ground plant material using 500 ml of hot 80% methanol over the course of 24 hours. The filtrate was again extracted using petroleum ether, ethyl ether, and ethyl acetate in that order. To guarantee full extraction, each procedure was repeated three times. Because it contained a lot of fatty compounds, the petroleum ether fraction was discarded, and the ethyl ether fraction (which contained free flavonoids) was recovered. The bound flavonoids in ethyl acetate fractions were investigated. Each fraction underwent a 2-hour hydrolysis in 7 percent H_2SO_4. After filtering the resulting combination, ethyl acetate was once more extracted from the filtrate. The ethyl acetate extract was extracted after being rinsed with distilled water until neutral. Both the free flavonoids and the bound flavonoids fractions of ethyl acetate were vacuum-dried, weighed, and kept in glass vials at 40° C until needed.

Determination of antioxidant activity

DPPH radical scavenging activity

Using the method described by Bhat and Karim (2009) [5], the 1, 1 - diphenyl 2 - picryl - hydrazil (DPPH) was used to assess the extracts' ability to scavenge DPPH radicals. Peel extract was aliquoted and combined with 3.9 ml of a 0.1

mM DPPH methanolic solution. After vigorous vortexing, the mixture was left in the dark for 30 minutes. At 515 nm, the absorbance was measured in comparison to a methanol blank. The scavenging activity of the radical was estimated using the formula:

$(Ab_{control} - Ab_{sample} / A_{control})$ 100.

Where $Ab_{control}$ is the amount of DPPH solution that is absorbed, and Ab_{sample} is the amount of DPPH solution that is absorbed after the sample has been added.

In order to determine the IC_{50} values, a linear graph of concentration vs. percent inhibition was created. Each sample's antioxidant activity was calculated using the inhibition curve and given as IC_{50}, which is the concentration needed to inhibit the generation of DPPH radicals by 50%. BHT (Butylated Hydroxy Toluene) (2.52 0.02) and ascorbic acid (2.54 0.00) were utilised as references.

Peroxidase assay (POX)

200 mg plant sample was homogenised in 10 ml of phosphate buffer, chilled for 20 minutes, and then centrifuged at 10,000 rpm. The enzyme extract was taken from the clear supernatant. With the following modifications, the activity was assessed using the methodology of Chance & Maehly (1955) [7]. In addition, 0.2 ml of water, 0.3 ml of pyrogallol, and 2.4 ml of phosphate buffer were added. Immediately after adding 0.1 ml of enzyme extract, the absorbance at 420 nm was measured to assess how much purpurogallin had produced. The enzyme activity, measured in mMoles per minute per gram dry weight, was calculated using the extinction coefficient of 2.8 $mM^{-1}cm^{-1}$.

Peroxidase value = O.D. value x 2.8 $mM^{-1}cm^{-1}$

Statistical analysis

The average of three analyses with standard deviation was used to express all experimental data, which were performed in triplicate. Additionally, linear regression analysis was used to get the IC_{50} values.

Results and discussion

Methanolic extracts of *C. angustifolia's* leaves, stem, and root which were collecetd from several locations in India were analysed for their total phenolic and flavonoid content. Since methanolic extracts were discovered to have significant levels of both TPC and TFC, studies on the antioxidant activity of these extracts have been conducted. These studies have included the DPPH assay and the POX enzyme assay. Although there have been a few reports on the *in vitro* DPPH assays of *C. angustifolia*, no research has been done on the species' methanolic extracts too far. Phenolics are significant secondary plant metabolites with known medicinal applications as antioxidants, anticarcinogens, and antimutagens. They have a history of lowering cardiovascular risks. The leaves from Rajasthan had the highest level of total phenolic content, which was found in the methanolic extract (21.98±3.65 mg GAE/gdw), followed by the leaves from Tamilnadu (20.98±1.65 mg GAE/gdw) (Table 1; Fig.1). The climate or plant environment is important which causes the variance in phenolic concentrations in the distinct places.

Table 1: Total phenolic, total flavonoids, and isolated flavonoid contents in *Cassia angustifolia vahl.*

Plant part	Locations	Total phenolic content (mg GAE/gdw)	Total flavonoid content (mg QE/gdw)	Isolated flavonoid (mg/d.dw)		
				Free (F)	Bound (B)	Total (F+B)
Leaves	R	21.98±3.65	38.86±11.09	2.134	1.296	3.397
	G	19.98±2.05	28.06±10.01	1.156	0.978	2.285
	T	20.98±1.65	33.12±09.02	2.101	1.002	2.976
Stem	R	12.47±8.84	23.87±9.32	1.074	1.523	1.597
	G	10.47±4.05	13.77±8.10	0.037	0.987	0.972
	T	11.47±6.36	19.34±7.02	1.001	1.002	1.203
Root	R	19.25±0.01	5.59±2.58	2.384	1.341	3.345
	G	16.25±0.03	3.12±1.06	1.196	0.149	2.540
	T	17.25±0.02	4.38±2.08	2.003	1.011	3.009

Note: R-Rajasthan, G- Gujrat, T- Tamilnadu. All experiments were done in triplicates. All values are presented as Mean±standard deviation.

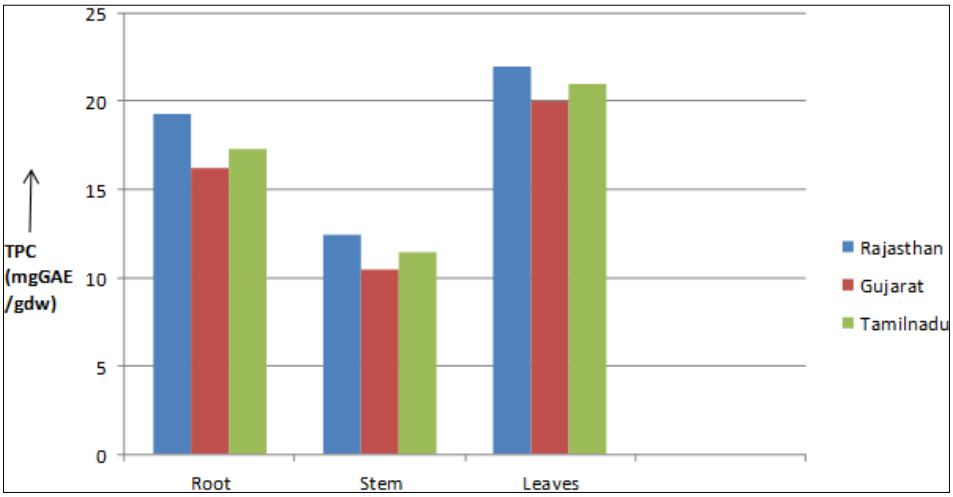

Fig 1: Total Phenolic Content (mgGAE/gdw) of *Cassia angustifolia* Vahl.

The class of polyphenolic chemical known as flavonoids includes substances that have powerful bioactivities against cancer, inflammation, ulcers, hepatotoxicity, allergies, and viruses. They are strong antioxidants because their phenolic hydroxyl groups enable them to efficiently scavenge ROS. The leaves collected Rajasthan had the highest levels of flavonoids (38.86±11.09 mg QE/gdw), followed by the leaves gathered in Tamilnadu (33.12±09.02 mg QE/gdw) (Table 1; Fig.2 and Fig.3). The extraction method and the plant's environment both affect how much flavonoid is present overall in plant extracts. High phenolic and flavonoid content in the extracts may have directly influenced the species' antioxidant activity. Given that plants with high flavonoid content can act as good sources of antioxidants to help increase an organism's overall antioxidant capacity and provide protection from lipid peroxidation, the presence of good amounts of phenolic and flavonoid contents demonstrated that both species are good sources of natural antioxidants (Sharififar *et al*., 2009) [27].

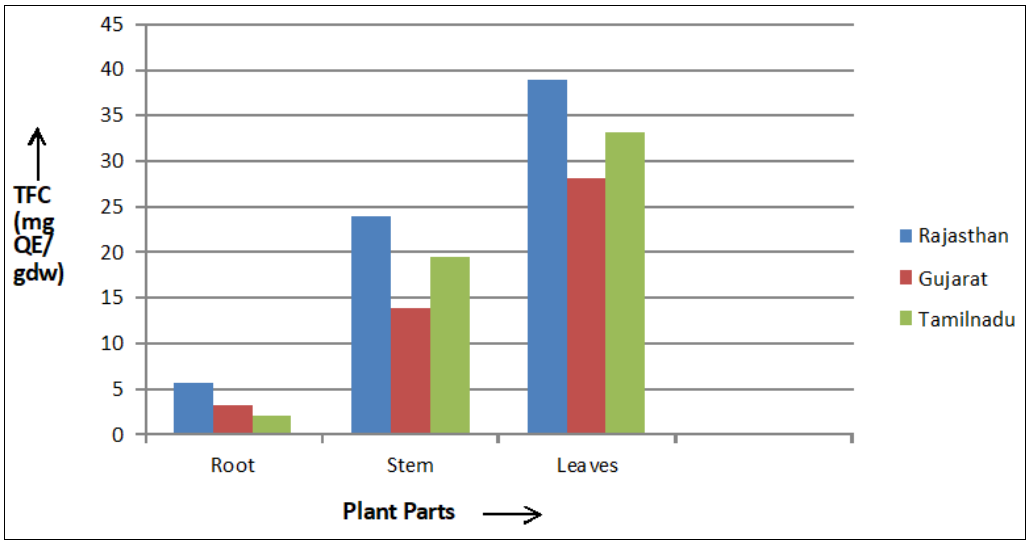

Fig 2: Total Flavonoid Content (mgQE/gdw) of *Cassia angustifolia* Vahl.

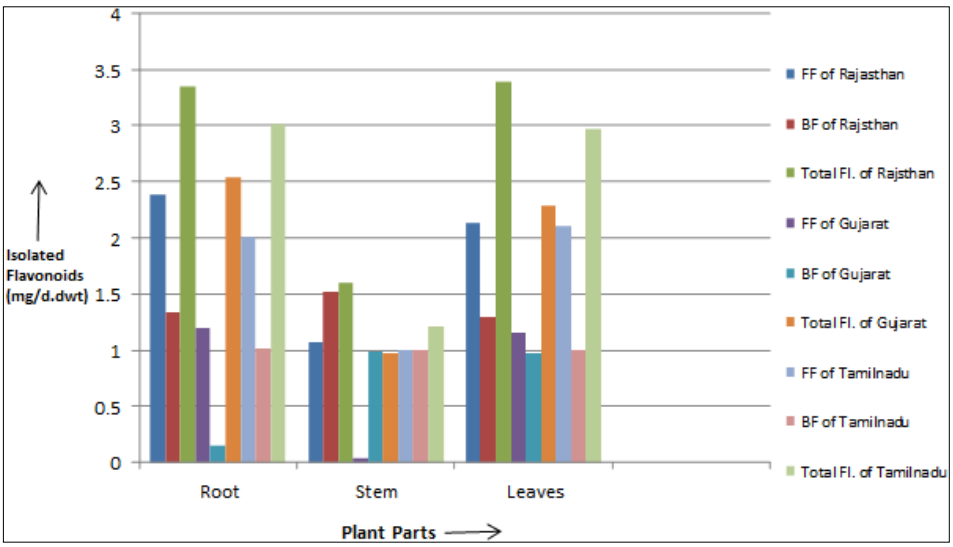

Fig 3: Isolated Flavonoids (mg/d.dwt) of *Cassia angustifolia* Vahl.

The most popular technique for assessing the antioxidant activity of natural antioxidants is DPPH. According to Janaszewska *et al.* (2002) [14], different antioxidants react with DPPH at varying rates, which affects how well they scavenge free radicals. Methanolic plant extracts may have scavenging potential since there are free hydroxyl groups available. Our findings also imply that there are more free hydroxyl groups in leaf methanolic extracts since stem extract has the highest DPPH free radical scavenging activity, followed by root and leaf extracts. The most effective plant parts for antioxidant potential were collected from Rajasthan (IC_{50}=98.309.03 mg/L [Leaves], 285.910.04 mg/L [Stem], 109.506.02 mg/L [Root]), followed by Tamilnadu (IC_{50}=100.108.01 mg/L [Leaves], 302.310.87 mg/L [Stem], 129.609.76 mg/L [Root]), and Gujarat (IC_{50}= 111.4±10.05 mg/ml [Leaves], 320.4±11.35 mg/L [Stem], 135.7±09.25 mg/L [Root]) (Table 2; Fig. 4). Leaf part of all location found to be more potent antioxidant than root and stem. In general, the antioxidant activity will be higher the lower the IC_{50} values. The discrepancy in their IC_{50} values indicates that the scavenging activity of the methanolic extract of the root is less than that of the leaves. The different polarity and solvent extract dissolving capacities amongst them may be the cause of the variation in their scavenging abilities. By using a one-way ANOVA analysis, no discernible difference was detected between the IC_{50}

values of in similar plant parts collected from various locations however, in a single plant, different parts showed varied antioxidant potential significantly. The potential antioxidant activity of a substance is indicated by its capacity to reduce power. The quantity of reductones a substance has determines how much it can reduce. These reductones block the chain of free radicals by giving a hydrogen ion. Higher amounts of reductones were found in the extracts as evidenced by the reducing power ability of methanolic extracts, which rose as the concentration of the test extract increased. The irreparable harm caused by oxidative stress depends on the production of OH* radicals. Hydroxyl radicals are produced by the Fenton reaction and are very reactive in biological systems. You can calculate a test compound's ability to scavenge hydroxyl radicals by looking at how free radicals affect deoxyribose's ability to degrade (Subhashini *et al*., 2011) [31].

Table 2: Evaluation of antioxidant activity of plant parts by DPPH assay

Name of plant part	Locations	IC_{50} (mg/L)
		Cassia angustifolia vahl.
Leaves	R	98.3±09.03
	G	111.4±10.05
	T	100.1±08.01
Stem	R	285.9±10.04
	G	320.4±11.35
	T	302.3±10.87
Root	R	109.5±06.02
	G	135.7±09.25
	T	129.6±09.76

Note: R-Rajasthan, G- Gujrat, T- Tamilnadu. All experiments were done in triplicates. All values are presented as Mean±standard deviation.

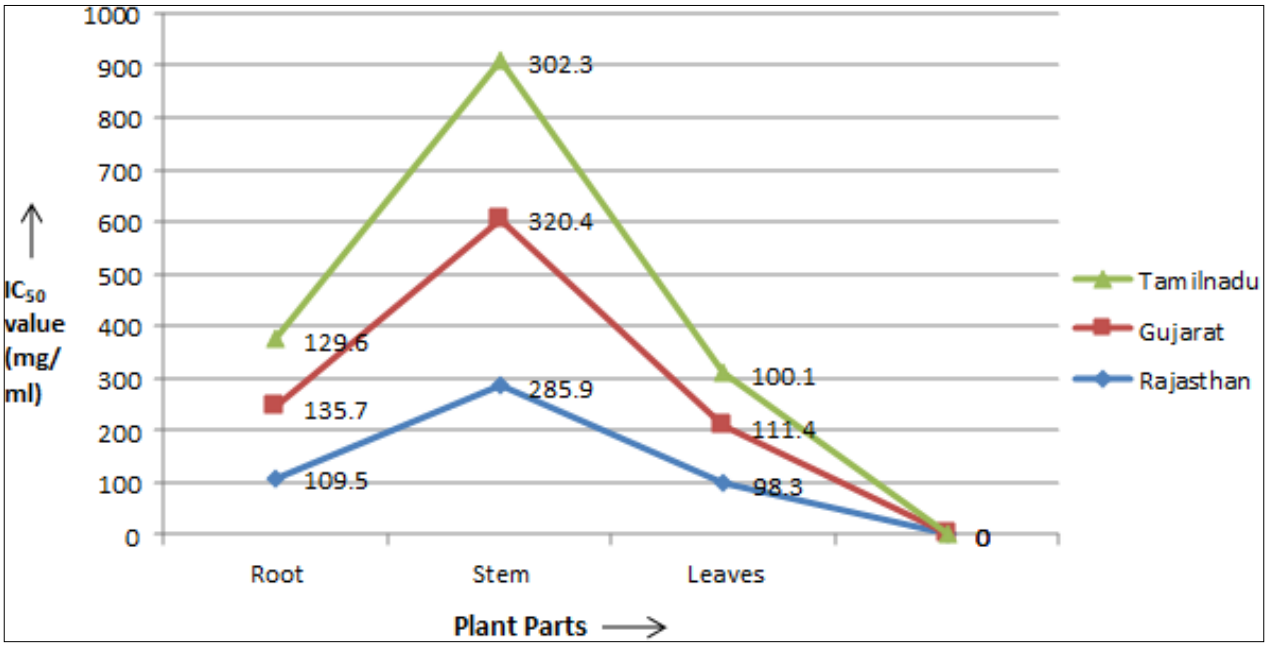

Fig 4: Evaluation of antioxidant activity of different parts of *Cassia angustifolia* vahl. By DPPH assay

The results of the DPPH assay were further corroborated by the peroxidase enzyme assay, which showed that leaves have more antioxidant potential than roots. More effective extracts came from Tamilnadu (0.246 0.022 mMole/min/g.dw [Leaves], 0.153 0.020 mMole/min/g.dw [Stem], 0.211 0.120 mMole/min/g.dw [Root]), Rajasthan (0.198 0.019 mMole/min/g.dw [Leaves], 0.131 0.017 mMole/min/g [Stem], 0.203±0.119 mMole/min/g.dw [Root]) and Gujarat (0.083±0.011 mMole/min/g.dw [Leaves] 0.076±0.010 mMole/min/g.dw [Stem], 0.108±0.108 mMole/min/g.dw [Root]). Roots were discovered to be more powerful than leaves and stems among all plant parts (Table 3; Fig. 5).

Table 3: Evaluation of antioxidant activity by peroxidase (POX) enzyme assay

Name of plant part	Locations	Peroxidase activity (mMole/min/g.dw)
		Cassia angustifolia vahl.
		Methanolic extract
Leaves	R	0.198±0.019
	G	0.083±0.011
	T	0.246±0.022
Stem	R	0.131±0.017
	G	0.076±0.010
	T	0.153±0.020
Root	R	0.203±0.119
	G	0.108±0.108
	T	0.211±0.120

Note: R-Rajasthan, G- Gujrat, T- Tamilnadu. All experiments were done in triplicates. All values are presented as Mean±standard deviation.

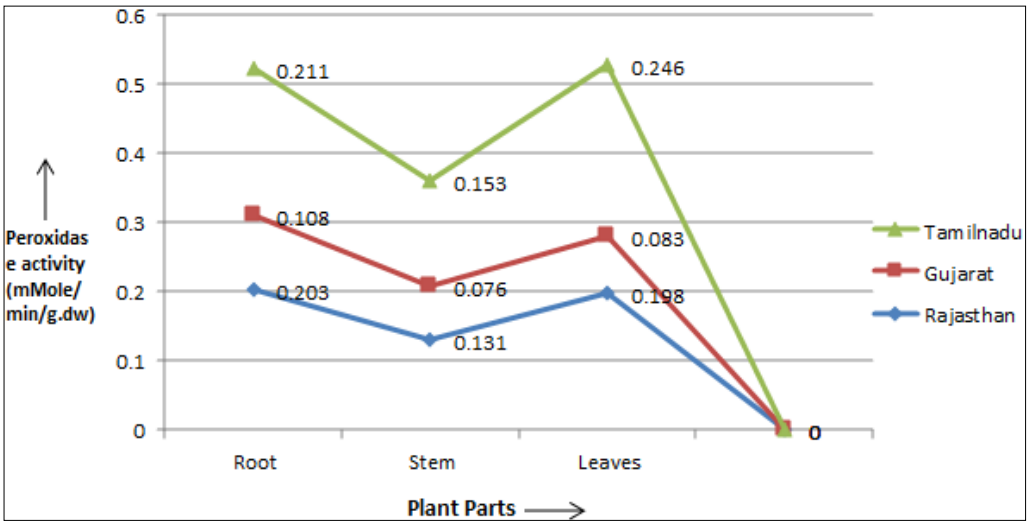

Fig 5: Evaluation of peroxidase enzyme activity of different parts of *Cassia angustifolia* vahl.

As a result, we can conclude that in the current study, methanolic extracts of leaves, roots, and stems collected from Tamilnadu showed more potent antioxidant activity than those from Rajasthan and Gujarat, while Rajasthan had the highest levels of phenolic and flavonoid content, followed by Tamilnadu and Gujarat.

Conclusion

The current study demonstrates that different parts of *Cassia angustifolia* vahl have significant flavonoid and phenolic content. The study's findings also indicated that these plant parts possess a significant amount of antioxidant compounds. Gujarat and Tamilnadu are followed in antioxidant activity and bioactive constituent richness in plant parts collected from Rajasthan. The results of the current study may help scientists and researchers in formulating plans for creating food crops high in antioxidants. Further research is needed to fractionate the extract, identify the bioactive compound(s), and conduct *in vivo* tests on its medicinal active ingredients in order to pinpoint their precise mechanisms of action, enhance nutritional benefits, and create high-quality natural pharmaceutical products.

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