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Phytochemical Analysis and *In Vitro* Antioxidant Activity of *Rasagandhi Mezhugu*, A Herbo-mineral *Siddha* Formulation

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Abstract

Background: The *Siddha* system of medicine is one of the ancient traditional medical systems that originated in India. *Rasagandhi Mezhugu* (RGM) is a herbo-mineral *Siddha* formulation which has 48 ingredients, including herbs, minerals and heavy metals. Though the therapeutic application of RGM is diverse, its phytochemical profile and antioxidant potential of RGM are not yet documented. Hence, the present study aimed to report these attributes for the Aqueous Extract of RGM (AE-RGM). **Methods**: AE-RGM was subjected to phytochemical analysis using standard procedures. The antioxidant capacity of AE-RGM was evaluated by using DPPH radical scavenging assay, superoxide radical scavenging assay, hydroxyl radical scavenging assay and nitric oxide radical scavenging assay. Finally, the Pearson correlation (r) coefficient was also calculated in order to elucidate the relationship between its phytochemical content and antioxidant potential. **Results**: Phytochemical analysis reveals the presence of various phytoconstituents. Quantitative phytochemical analysis revealed the presence of 5.55±0.40 mg of Gallic Acid Equivalent (GAE/g) of total phenols, 0.098 ± 0.049 mg of Quercetin Equivalents (QE/g) of total flavonoids, 0.067 ± 0.05mg of GAE/g of tannins and 0.141 ± 0.05mg/g of alkaloids in AE-RGM. Radical scavenging assays have demonstrated the IC_{50} value of about 97.9µg/ml, 142.5µg/ml, 901µg/ml, 571µg/ml for DPPH, Superoxide, Nitric oxide and hydroxyl radicals respectively. A strong, significant positive correlation ($p<0.05$) was also found between phenols, flavonoids, tannins and all the antioxidant activities except the nitric oxide scavenging assay, which exhibited moderate correlation. **Conclusion:** The present study evidenced and validated the antioxidant nature of RGM, which might be due to its rich phytochemical content. This renders RGM with therapeutic potential against free-radical-associated oxidative damage. The study also adds scientific validation to the clinical practice of RGM for various benign and malignant tumours. Further research is needed to elucidate its therapeutic mechanisms at the cellular level to validate its clinical application, as mentioned in *Siddha* classical texts.

Major Findings: *Rasagandhi Mezhugu* (RGM), a traditional *Siddha* formulation, exhibits significant antioxidant properties. RGM contains a variety of phytochemicals, including phenols, flavonoids, tannins, and alkaloids, which contribute to its antioxidant activity. This study provides scientific evidence supporting the traditional use of RGM and its potential benefits in preventing oxidative damage.

Keywords: Antioxidant Activity, Phytochemical Analysis, *Rasagandhi Mezhugu* (RGM), Traditional Medicine

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1. Introduction

The traditional system of medicine employs over 6000 species, a significant contrast to the 3000 formally recognised plants with medicinal properties[1]. Within the context of the body's integrated antioxidant system, various types of free radicals are typically generated at low levels within cells to assist in regulating multiple physiological functions. However, an excess of free radicals can be detrimental, potentially leading to conditions such as ischemia, lung damage, inflammation, and other degenerative disorders. Numerous phytochemicals and plant extracts, especially phenolic compounds like flavonoids and benzyl iso thiocyanate, have demonstrated their ability to act as antioxidants and effectively scavenge free radicals[2]. Notably, more than 60% of officially approved medications have origins in natural sources, with a significant portion of these discoveries originating from traditional herbal remedies.

Siddha, a prominent ancient medical system in India, is believed to have originated from 10000 - 4000 B.C[3]. The uniqueness of the *Siddha* system is evident in its continuous service to mankind for ages in combating diseases and in maintaining its physical, psychological and social well-being. *Siddha* system of medicine provides preventive, promotive, personalised, curative, rejuvenative and rehabilitative treatment modalities by following a scientific and holistic approach[4].

RGM is a herbo-mineral *Siddha* formulation composed of 38 distinct botanicals and 8 inorganic compounds, including certain heavy metals[5]. As per *Siddha* literature, RGM has been indicated for various ailments, including *Soolai* (Pain), *Kandamaalai* (Cervical lymphadenitis), *Kiranthi* (Glandular swelling), *Vippuruthi* (Tumour), *Kurai noi* (Leprosy), *Mega rogangal* (Urogenital disorders), *Puttru noi* (Cancer), *Pilavai* (Carbuncle), and *Pautthiram* (Fistula)[6]. It is commonly used for cancer, benign tumours, venereal diseases, skin diseases, and neurological ailments. *Siddha* practitioners prescribe RGM as a therapy for different types of cancers. Some of the studies proved the anti-cancerous activity of RGM in an animal model[7-9].

Despite its usage in *Siddha* practice, there exists insufficient data to conclusively support its phytochemical contents and antioxidant nature. Therefore, the primary objective of this study is to comprehensively explore the phytochemical and antioxidant attributes inherent in RGM. The outcome of this study provides empirical evidence and scientific validation that unequivocally supports its anti-proliferative efficacy.

2. Materials and Methods

2.1 Study Drug

RGM was obtained from the Indian Medical Practitioners Co-operative Pharmacy and Stores Ltd. (IMPCOPS), Thiruvanmiyur, Chennai, India. Its batch number was S II – 206.

2.2 Chemicals

Ascorbic acid, Butylated hydroxytoluene, Gallic acid, Curcumin, Trichloroacetic acid, 2,2-diphenyl 1-picrylhydrazyl radical (DPPH), Sodium phosphate, Ferrous ammonium sulfate, Ammonium molybdate, Ethylene Diamine Tetra Acetic Acid (EDTA), Dimethyl Sulfoxide (DMSO) and all other chemicals were procured from LOBA Chemie Pvt Ltd., Mumbai, SRL, Mumbai and Merck-India, Bangalore. All chemicals and solvents used in the experiments were of analytical grade.

2.3 Extraction

This was done by the cold percolation method. 50g of RGM was soaked in 250ml water, and the mixture was kept at room temperature for 72 hours. After that, the mixture was filtered using Whatman No.1 filter paper. The filtrate was evaporated to dryness and stored at 4°C for further analysis.

2.4 Phytochemical Analysis

The aqueous extract of RGM (AE-RGM) was subjected to the phytochemical analysis as described by Sofowara[10], Trease and Evans[11], and Harborne[12] to confirm the presence of various phytochemicals.

2.5 Estimation of Phytochemicals in RGM

Total phenols estimation was performed by following the method of McDonald *et al.*,[13], and the determination of total flavonoids content in the AE-RGM was carried out using the procedure of Chang *et al*[14]. Total tannins content in AE-RGM was quantified according to the

procedure of Peri and Pompi[15]. To estimate the alkaloid content, the methodology of Harborne[16] was employed.

2.6 Free Radical Scavenging Activity of RGM

A modified method of Blois was adopted for DPPH assay[17,18]. Superoxide anion radical scavenging activity was determined according to Nishimiki *et al*[19]. To evaluate the Nitric oxide scavenging activity of AE-RGM, the method described by Jagetia *et al.*,[20] was employed. The ability of AE-RGM aqueous to scavenge hydroxyl radicals was evaluated using the method outlined by Kalaisezhiyan *et al*[21]. The percentage of scavenging in all the assays was calculated as follows:

$$\text{Percentage of Scavenging} = (A_{control} - A_{sample}) / A_{control} \times 100$$

Where A_{sample} is the absorbance of the test sample (aqueous extract of RGM), and $A_{control}$ is the absorbance of the control.

2.7 Statistical Analysis

All the analyses were done in triplicate. The values were expressed as Mean ± Standard Deviation. Pearson correlation (r) analysis was done to demonstrate the correlation between various phytochemicals and free radical scavenging activity. All statistical analyses, including IC_{50} value determinations, were performed using GraphPad Prism software (9.3.1).

Table 1. Qualitative phytochemical analysis of AE-RGM

Sl. No.	Name of the test	Aqueous Extract of *Rasagandhi Mezhugu* (AE-RGM)
1	Alkaloids	+
2	Coumarins	+
3	Tannins	+
4	Saponins	-
5	Flavonoids	+
6	Quinones	+
7	Phenols	+
8	Carbohydrates	-
9	Proteins	-
10	Cardiac Glycosides	-
11	Steroids	-
12	Starch	-

3. Results

3.1 Qualitative Phytochemical Analysis

The Qualitative phytochemical analysis of aqueous extract of RGM (AE - RGM) for various phytochemicals revealed the presence of alkaloids, coumarins, tannins, flavonoids, quinones and phenols (Table 1).

3.2 Quantitative Analysis

Major phytochemicals, including total phenols, total flavonoids, total tannins and alkaloids whose presence was observed in qualitative phytochemical analysis, were quantified in AE-RGM. All the results were expressed as mean ± SD.

Based on the standard regression lines of Gallic Acid (R^2=0.9878) and Quercetin (R^2= 0.9992), the equivalents of standards, i.e. mg of GAE per gram of extract and mg of QE per gram of extract, were calculated. The total phenol content of RGM extract was found to be 5.55 ± 0.40 mg of GAE/g, and the total flavonoid content was found to be 0.098 ± 0.049 of QE/g. The aqueous extract of RGM was found to contain an appreciable amount of tannins. It contains 0.067 ± 0.05 mg of GAE/g. AE-RGM also contains 0.141 ± 0.05 mg/g of alkaloids (Table 2).

3.3 Antioxidant Activity

The antioxidant potential of the aqueous extract of RGM was assessed through various methods, including DPPH radical scavenging activity, Superoxide anion radical scavenging activity, Nitric oxide radical scavenging activity, and Hydroxyl radical scavenging activity. AE-RGM has exhibited dose-dependent activity towards the free radical inhibition.

3.3.1 DPPH Scavenging Activity

The aqueous extract of RGM exhibited an incremental increase in its scavenging activity as the concentration of the extract increased. RGM demonstrates potential

Table 2. Quantitative analysis of phytochemicals in RGM

Sl. No.	Phytochemicals	AE - RGM
1	Total polyphenols in mg of GAE/g	5.55 ± 0.40
2	Total Flavonoids in mg of QE/g	0.098 ± 0.049
3	Total Tannins in mg of GAE/g	0.067 ± 0.05
4	Alkaloids in mg/g	0.141 ± 0.05

DPPH radical scavenging properties, with values ranging from 44% to 73% with an IC_{50} value of 97.9µg/ml when compared to the standard Butylated hydroxyl toluene, which displayed percentage inhibition ranging from 65% to 94% with an IC_{50} value of 47 µg/ml (Figure 1).

3.3.2 Superoxide Radical Scavenging Activity

The antioxidant activity of AE-RGM demonstrates a dose-dependent rise as the concentration of the extract increases gradually. AE-RGM exhibits significant scavenging properties against superoxide anion radicals, with values ranging from 35.8% to 87.2%, and with an IC_{50} value of 142.5 µg/ml. Whereas, the standard ascorbic acid has shown a percentage inhibition ranging from 48.2% to 79.4%, with an IC_{50} value of 52 µg/ml (Figure 2).

3.3.3 Nitric Oxide Radical Scavenging Activity

Figure 3 depicts the nitric oxide radical scavenging activity of AE-RGM. The AE-RGM also demonstrated the potential for scavenging nitric oxide radicals, with percentages ranging from 32% to 55% and an IC_{50} value of 901 µg/ml. Standard curcumin has shown inhibition percentages ranging from 9% to 69% with an IC_{50} value of 66 µg/ml. From this result, it was evident that AE-RGM has a moderate capacity to scavenge nitric oxide radicals.

3.3.4 Hydroxyl Radical Scavenging Activity

The scavenging action of AE-RGM exhibited a dose dependency on hydroxyl radicals, showing a gradual increase in a concentration-dependent manner. RGM demonstrates noteworthy hydroxyl radical scavenging capabilities, with values ranging between 56% and 66%. The IC_{50} value for AE-RGM was found to be 571.8 µg/ml. Gallic acid, the standard, has exhibited percentage inhibition ranging from 40% to 67%, with an IC_{50} value of 61 µg/ml (Figure 4).

3.4 Correlation Analysis

In the interest of understanding the relationship between phytochemical contents and free radical scavenging activity of AE-RGM, correlation coefficients (r) and coefficient of determination were estimated. Strong correlations were demonstrated between the antioxidant nature and total phenols, flavonoids and tannins (Figure 5a, b, c), indicating that these phytochemicals, present in RGM, contribute to the inhibition of oxidation reactions. Table 3 shows the Pearson correlation coefficient (r) and coefficient of determination obtained for various phytochemicals against free radical scavenging assays. Among the radical scavenging assays, DPPH and superoxide anion radical scavenging assays have a strong correlation ($P<0.0001$) with both total phenol and total flavonoid content. The hydroxy radical scavenging assay correlates significantly with total phenol and tannins ($P<0.0001$); lesser significance was observed for total flavonoid content($P<0.001$). A stronger correlation was confirmed between phenols, tannins and hydroxyl radical inhibition. Comparatively less significant correlation was identified between the nitric oxide scavenging assay and all three phytochemical contents ($P<0.001$).

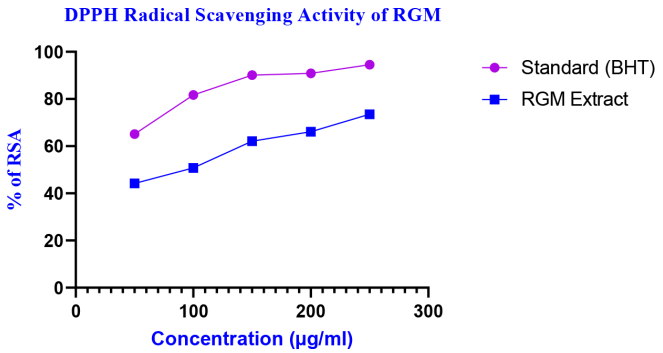


Figure 1. DPPH radical scavenging activity of RGM.

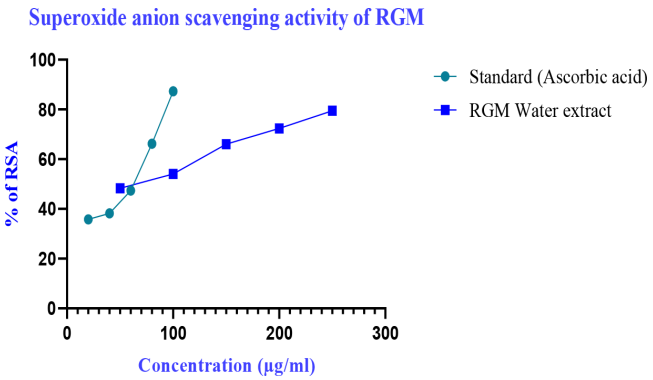


Figure 2. Superoxide anion radical scavenging activity of RGM.

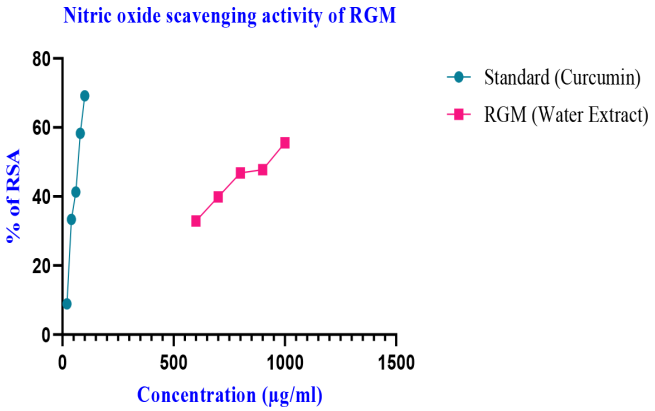


Figure 3. Nitric oxide radical scavenging activity of RGM.

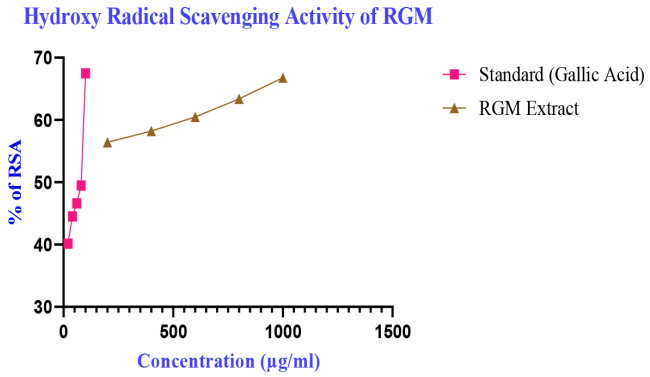
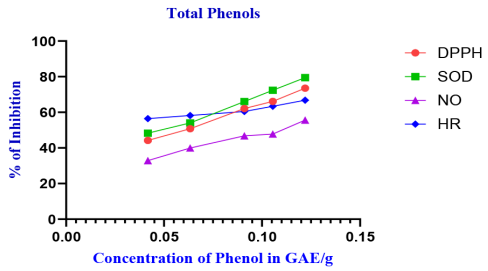


Figure 4. Hydroxyl radical scavenging activity of RGM.

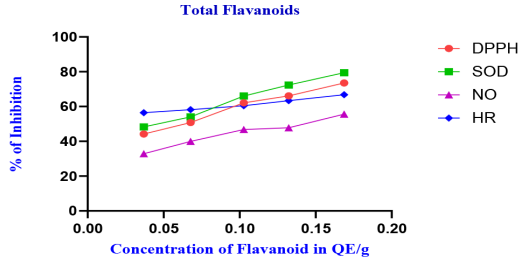
Table 3. Correlation analysis of various phytochemicals and free radical scavenging activity. R, correlation coefficient; R2, coefficient of determination. *** represents significance level at P<0.0001, ** represents significance level at P<0.001

S. No.	Free Radical Scavenging Activity	Total Phenols		Total Flavonoids		Total Tannins	
		R	R^2	R	R^2	R	R^2
1	DPPH scavenging activity	0.9981***	0.9962	0.9924***	0.9849	0.9775**	0.9554
2	Superoxide anion radical scavenging activity	0.9966***	0.9933	0.9942***	0.9885	0.9837**	0.9678
3	Nitric oxide radical scavenging activity	0.9864**	0.9729	0.9836**	0.9675	0.9659**	0.9329
4	Hydroxyl radical scavenging activity	0.9728**	0.9463	0.9935***	0.9871	0.9969***	0.9938

5 (a)



5 (b)



5 (c)

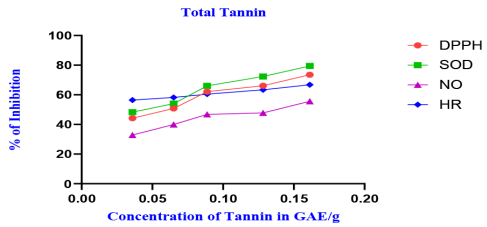


Figure 5. Correlation analysis of phytochemicals and DPPH, superoxide anion radical (SOR), nitric oxide radical(NO), and hydroxy radical (HR). This figure illustrates the correlation analysis between various phytochemicals (z-axis) and % of inhibition (y-axis). **(a)**The correlation of total phenol vs free radicals, **(b)** Flavanoids vs free radicals, **(c)** Tannins Vs free radicals.

4. Discussion

Traditional medicines, with their rich repertoire of phytochemicals, offer a promising avenue for drug discovery. This manuscript delves into the exploration of RGM, a classical *Siddha* formulation, known for its therapeutic efficacy against various ailments. RGM has been in safe clinical practice for many hundreds of years without any notable adverse effects for the management of uterine fibroid (*Pulippani Vaithiyam* -500[22]). This herbo-mineral formulation is included in "The Siddha Formulary of India", Part I (English), 1992, which was one of the enlisted books under the Drugs and Cosmetics Act, 1940 (The Siddha Formulary of India, Part I, 1992)[5]. Uterine fibroids are tumours of smooth muscle cells and fibrous connective tissue, which are of a benign type[23]. During clinical practice, many *Siddha* physicians have observed fibroid shrinkage after the intervention of RGM successfully. The synergistic effects of various ingredients present in RGM have also revealed its anti-proliferative potency in the management of uterine fibroids[24]. A clinical trial (open pilot clinical trial with a sample size of 20) was also conducted in the non-surgical management of fibroid uterus, with RGM found to be beneficial[5]. Based on its results, a multi-centric clinical trial with a larger sample size is underway in four different centres of the Central Council for Research in Siddha (CCRS) institutes/units to scientifically validate the effectiveness of RGM in fibroid uterus patients.

The present study is an extended work of the above clinical study, which provides experimental evidence for its anti-proliferative and antioxidant activity. Previously, standardisation of RGM was performed, and it explains the physicochemical characteristics, heavy metal content and High Performance Thin Layer Chromatography (HPTLC) fingerprinting of RGM[5]. In the present study, qualitative phytochemical analysis of RGM revealed the presence of alkaloids, coumarins, tannins, flavonoids, quinones, and phenols. These bioactive compounds are well-known for their antioxidant properties and have been extensively studied for their potential anticancer effects. Furthermore, quantitative analysis demonstrated significant concentrations of total phenols, total flavonoids, total tannins, and alkaloids in RGM, indicating its potential therapeutic value. Tannins are polyphenolic compounds, and they are abundant in plant sources. Tannins have enormous activity against haemorrhage, diarrhoea, and hemorrhoids also have potent anti-cancer and cytotoxic activity[26]. Tannins have demonstrated antimicrobial activity against various viruses, bacteria, fungi and parasites[26]. Phenols and flavonoids also have antioxidant, anti-inflammatory and anti-cancer activity[27]. Alkaloids are reported to have a great effect on neurological disorders like Alzheimer's disease[28] and also have been demonstrated for anticancer activities[29].

Diverse antioxidant assays have been reported with various mechanisms of action to evaluate and prove the presence of antioxidant potential of plants. These assays validate the antioxidant nature of plants in diverse ways before certifying it. The antioxidant activity of RGM was assessed through various assays, including DPPH radical scavenging assay, Superoxide anion radical scavenging assay, Nitric oxide scavenging assay and Hydroxyl radical scavenging assay.

The presence of phenolics and flavonoids provides the free radical scavenging capabilities to the medicinal plants. Phenols and flavonoids are very much extractable in polar solvents, and they possess good scavenging potential as they donate electrons or hydrogen to stabilise DPPH free radicals. Oxidative enzymes produce superoxide anion by non-enzymatic reaction, such as autooxidation by catecholamines from molecular oxygen[30,31]. Superoxide anions indirectly initiate lipid oxidation and serve as precursors of singlet oxygen and hydroxyl radicals[32]. Nitric Oxide (NO) is an important chemical mediator which is involved in the regulation of various physiological processes[33]. NO is generated by various cell types, such as endothelial cells, macrophages, and neurons. Accumulation of NO is associated with the pathogenesis of several diseases[34]. Free radicals like nitrite and peroxynitrite anions are generated from nitric oxide by reacting with oxygen[35]. These reactive species play an important role in mediating many genotoxic effects, such as DNA damage. Nitric oxide reacts instantly in the intracellular environment to form reactive nitrogen species like nitrate, nitrite and S-nitrosothiols. NO causes DNA damage via peroxynitrite. Hydroxyl radical can react with nucleotides in DNA, thereby causing strand breakage, which in turn leads to carcinogenesis, mutagenesis, and cytotoxicity. It is a very toxic and

short-lived free radical that initiates a chain reaction that damages the cellular integrity[36]. Besides, hydroxy radicals are considered to be one of the fastest initiators of the lipid peroxidation process by abstracting hydrogen atoms from unsaturated fatty acids. RGM exhibited potent antioxidant properties in all assays, with dose-dependent scavenging activity comparable to standard antioxidants like Butylated Hydroxytoluene (BHT), ascorbic acid, curcumin, and gallic acid. These findings underscore the potential of RGM as a natural antioxidant agent, capable of neutralising harmful free radicals implicated in cancer initiation and progression. The observed antioxidant activity suggests its potential role in mitigating oxidative stress by scavenging free radicals and restoring redox balance.

The relationship between the antioxidant activity and the phytochemical content of traditional medicines or medicinal plants can be due to many factors, viz., total phenolic content, total flavonoid content and total tannin content. In addition, the synergistic effect of various antioxidants in the formulation also confers the antioxidant activity. This kind of effect not only depends on the concentration, but also depends on the structure and the type of interaction that exists between the antioxidant phytochemicals. The Pearson correlation (r) is used to determine the degree of the linear relationship between any two variables. In the present study, this correlation was determined to estimate the type of relationship that exists with the various phytochemicals and antioxidant assays. Many studies have demonstrated that the phenolic compounds are the major antioxidant components in various herbs, spices, vegetables and fruits, and there exists a direct relationship with the antioxidant activity and total phenolic content[37,38]. The highly significant positive correlations obtained in this study for total phenolic content and total flavonoid content support the antioxidant capacity of RGM. This finding is in accordance with the findings of Cai *et al.*,[39] and Djeridane *et al.*,[40], who observed a linear correlation between the total phenol content and antioxidant capacity of several plant species. Among the three antioxidant assays, a less significant relationship was found between the nitric oxide scavenging assay and all three phytochemicals. The hydroxyl radical assay has shown a stronger correlation with tannin content than the phenolic and flavonoid contents. All these results together validate the antioxidant nature of AE-RGM. As the anti-proliferative and anti-inflammatory pathways involve oxidative stress in their initial stages, the antioxidant nature of RGM scientifically validates its therapeutic usage for various benign and malignant tumours.

5. Conclusion

This study provides scientific evidence supporting the antioxidant properties of RGM, a classical *Siddha* formulation. Through phytochemical analysis, RGM revealed a rich composition of bioactive compounds known for their antioxidant activity, suggesting its capacity to mitigate oxidative stress, a key factor in cancer development. Further research is required to elucidate the molecular mechanisms underlying its anticancer effects and also to explore its therapeutic effectiveness in clinical trials.

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Author Contribution

GG – Conceptualisation, Methodology, Validation, Data Curation and Writing—Original Draft Preparation. SR – Conceptualisation, Resources, Data Curation and Writing—Review and Editing. RK- Resources, Visualisation, and Supervision. AA- Investigation, Software, Formal Analysis and Writing—Original Draft Preparation. SS- Resources, Visualisation, and Supervision. NJM- Resources, Visualisation, and Supervision. All authors have read and agreed to the published version of the manuscript.