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PHYTOCHEMICAL PROFILING AND IN-VIVO ANTIOXIDANT SCREENING OF GREWIA ASIATICA LEAVES EXTRACTS

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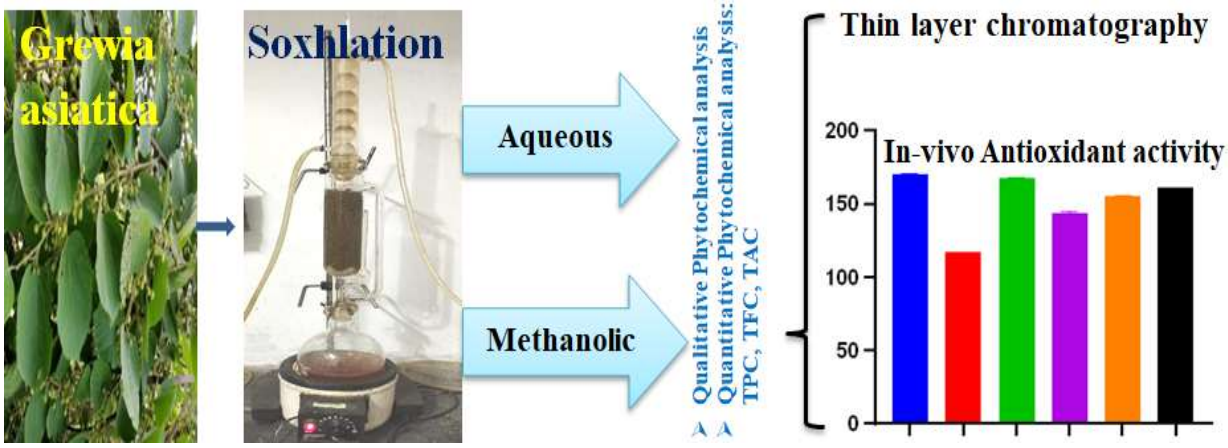
GRAPHICAL ABSTRACT



ABSTRACT

Background: Grewia asiatica is a berry plant, commonly called phalsa of Malvaceae family. The literature suggests that the plant is popular in folk medicines.

Aim: The study aimed to explore the pharmacognostical and radicle scavenging profile of Grewia asiatica leaves.

Methodology: Study uses a multidimensional methodology to understand the Phytochemistry and analyze its medicinal potential. For this design, plant morphology, physiochemical properties, preliminary TLC, SOD (Superoxide dismutase), GSH (glutathione) and LPO (Lipid peroxidation) methods were employed.

Result and Discussion: The study indicates that the plant has cordate leaves with pointed apex. The percentage yield of aqueous and methanolic extract was 33.238gms and 15.335gms, respectively. Preliminary phytochemical study verified carbohydrate, protein, tannins, and glycosides, along with considerable concentration of flavonoid, phenol and alkaloid in both extracts, highly in methanolic extract. TLC of methanolic extract confirms the presence of crucial constituents, prioritizing the molecular heterogeneity. In the CCl_4 treated animals, the significantly induction of oxidative stress was demonstrated in 28 days study. The treatment animals with 100, 200 and 400mg/kg/day G. asiatica methanolic leaves extract are evidenced to balance free radicles by reduced LPO, increased intracellular GSH and SOD levels. The treatment in the dose of 200 and 400mg/kg was significantly produce similar results as produce by standard drug silymarin.

Conclusion: The study demonstrated the Pharmacognostical and therapeutic standards of Grewia asiatica with laying the foundation for the forthcoming medicinal investigation moreover, patient oriented investigation.

KEYWORDS: Antioxidant Activity, Chromatography, Grewia asiatica, Phytochemicals, Total flavonoid, Soxhlation.

1. INTRODUCTION

India is known for its wealthy heritage within the field of medicinal plants. Plants are used as an alternate therapies in varied diseases because of their effectiveness, less toxicity and low price **[1]**. The utilization of herbal drugs has risen remarkably in line with the world trend of individuals returning to natural therapies. The UN agency acknowledges that pharmacognostical standards ought to be proposed as a protocol for the identification of herbal drugs, and documented raw material should be the fundamental place to begin for developing a botanical product. The safety and efficacy of herbal merchandise are dependent upon their standardization **[2]**. G. asiatica L. is belonging to Malvaceae family and conjointly referred to as 'star apple' or 'Phalsa' **[3]**. It's native to India and the kingdom of Nepal. The species is popular for its nutritional & healing traits **[4]**. The word "L." cites to Carl Linnaeus (1707–1778), primarily depict the species **[5]**. Many therapeutic uses have been shown to be present in different parts especially in leaves and fruits of plant. The plant

has been scientifically verified to demonstrate insecticidal, larvicidal and free radical scavenging activities **[6]**. Phalsa were utilized in ancient medicines. The fruit have a refreshing, digestive & styptics properties. Cortex concoction is palliative, antipyretic and effective to manage loose stools. The pustural blisters are cured from leaves. The rhytidome and roots are employed as a medication of arthritis **[7]**. The proposed investigation was tackled to standardize the Pharmacognostical and antioxidant properties of Grewia asiatica leaves.

2. METHODOLOGY

2.1 Chemicals

All reagents employed in this study were of laboratory grades, collected from Central Drug House, Himedia, Yacca enterprises and, Sigma Aldrich (India).

2.2 Plant Sample Procurement

Botanical specimen was identified by the literature survey and the common name. Plant leaves were collected from the Lakshar, District Haridwar (Uttrakhand), India and verified by the Botanical Survey of India (BSI), Dehradun, with specimen (number 705). The leaves of the documented plants were collected in sufficient quantity, washed with water, dried and powdered for further studies.

2.3 Macro-morphological and Physiochemical Analysis

The macroscopic traits (size, shape and surface) and organoleptic properties, including appearance, color, odor and taste of the crude leaves were evaluated **[8]**. Powdered leaves of G. asiatica were subjected to physiochemical analysis for their extraction value (Soxhlet extraction using aqueous and methanol as a solvent) **[9]**, pH value and solubility **[10]**.

2.4 Phytochemical Analysis

Both the extracts are subjected for qualitative analysis to detect various phytoconstituents present such as, alkaloid, flavonoids, carbohydrates, tannins, glycosides, proteins, phenols, steroids and triterpenoids via various standard methods **[11][12].**

2.5 Quantification of Total Phenolic Content

The FC procedure was used. Methanol and aqueous extract (0.2 ml) stocks of G. asiatica were mixed separately in test tubes with 3 ml of reagent, wait for 10 minutes. Add 2 ml Na_2CO_3 (7.5%), and increase volume to 7 ml with H_2O (dist., deionized), mixed thoroughly. Incubate the mixture in the dark for 90 minutes at 25°C, measure the λmax at 760 nm. The TPC (Total Phenolic Content) was calculated by the calibration curve extrapolation method, with a gallic acid solution (20-100 µg/ml). The procedure is repeated in thrice. The TPC was mentioned as mg GAE/g of sample **[13]**.

2.6 Quantification of Total Flavonoid Content

The Aluminium chloride procedure was used. Methanol and aqueous extract (0.5 ml) stocks of G. asiatica were mixed with 0.15 ml aluminium chloride hexahydrate (10%) and 0.15 ml sodium nitrite (5%) in 10 mL test tubes separately. After 10 minutes, add 4% sodium hydroxide (2 ml), and increase volume to 5 ml with H_2O (distilled, deionized). Measure the λmax at 510nm. Standard curve generated with a rutin solution (20-100 µg/ml) according to the method described above. The TFC (Total Flavonoid Content) was reported as mg rutin equivalents/g of sample **[14]**.

2.7 Quantification of Total Alkaloid Content (TAC)

The amount identified by the Harbone method. A 100 ppm solution of the standard alkaloid was made by diluting 1 mg atropine (10 ml dH_2O). Then, 0.5, 1, 1.5, 2, and 2.5 mL of atropine, 5 ml phosphate buffer saline (pH 4.7) and Bromocresol green were added in a shaker. 5ml of tetrachloromethane was added to each sample. The extracts were increased to 10 ml with trichloromethane. λmax was analysed at 470 nm and calibration graphs were plotted **[15]**.

2.8 Thin Layer Chromatography (TLC)

To evaluate the chemical profile, methanolic extract spots were applied manually via glass capillaries on precoated silica gel-G (20×20, 60 F_{254}, 0.02cm thick) plates, 1 cm above the bottom and at equal distances along with standard Nuciferine **[16]**. For maximum band separation on plates, toluene:ethtyl acetate:acetic acid was used as a standard solvent system. The mobile phase was selected on the basis of a literature survey. The plates are placed in, a presaturated TLC chamber and allowed to run until the mobile phase runs approximately 1 cm from the top. Upon completion, the plates were air dried, and the chromatograms were analysed under visible, UV light (at 254nm and 365nm). The number of bands was marked and the retention factor (Rf) of each band was computed **[17]**.

$$\mathbf{Rf} = \text{Path length of solute/Path length of solvent front}$$

2.9 Experimental Animals

Thirty six male Wistar Rat, 10-12 weeks old, weighing 180-200g were kept in polypropylene cages, provided with water ad libitum and normal pellet diet throughout the study. The rats were allowed to habituate to the experimental surroundings, with 12/12-h day-night at 22 ± 2°C. The investigation got the certification from the Committee for the

Control and Supervision of Experiments on Animals (CCSEA) (Reg No. 1824/PO/ReBi/S/2015/CCSEA) and the Institutional Animal Ethical Committee of PBRI (Pinnacle Biomedical Research Institute), Bhopal **[18]**.

2.10 Experimental procedure

Randomly divide animals in 6 groups (n=6). Acute inflammation was established by administering carbon tetrachloride i.p. diluted in olive oil (1:9 v/v), semiweekly. The treatment (Grewia asiatica methanolic leaves extract, GAMLE) was administered orally once daily for a period of 28 days. The animals in Groups II to VI received CCl_4 (1 mL/kg body weight) i.p. t semiweekly for 28 days **[19][20]**.

a) I Group (Normal control, normal water).
b) II Group (Positive control, CCl_4).
c) III Group (Standard group, silymarin, 25 mg/kg + CCl_4).
d) IV Group (Treatment I, GAMLE, 100 mg/kg+ CCl_4)
e) V Group (Treatment II, GAMLE, 200 mg/kg+ CCl_4)
f) VI Group (Treatment III, GAMLE, 400 mg/kg+ CCl_4)

2.11 Determination of antioxidant activity

The Superoxide Dismutase (SOD) test (McCord method, 1994) employed triphenyl tetrazolium chloride for identification of oxide radicals generated by formazan dye. SOD quantify at 560nm **[21][22]**. Lipid peroxidation (LPO) was assayed by the Buege and Aust,1978 procedure **[23]**. GSH was analyzed using glutathione assay kits at 412 nm **[22]**.

2.12 Statistical

Findings provided as Mean ± SD (n=6), interpret statistically via ANOVA (one-way analysis of variance) observed through post-hoc test using Graph Pad Prism11.0, and Microsoft Excel. **p< 0.01, ***p< 0.001, ****p< 0.0001 vs positive control; #p< 0.001 vs normal control was considered as level of significance while comparison between groups.

3. RESULT AND DISSCUSSION

3.1 Macromorphology and Physiochemical Analysis

G. asiatica is a fruit bearing plant belonging to the Tiliaceae family. The leaves are broad, 4- 20 cm long, 8-15 cm in width, cordate, hairy, and have a pointed apex. The organoleptic properties of both the dried leaves and the extracts clearly reveled that, the appearance, taste, odor and color of both extract were similar but that the contents of the crude powders were different. The dried leaves (300gm) were extracted with methanol and water. A preliminary study revealed a greater yield in aqueous extract (33.238gm). similarly, sonawane demonstrated that leaves have the best extraction yield in aqueous, followed by alcohol **[6]**. Both the extract was weak acids with pH ranging from 5.42 to 6.34. The results revealed that the methanolic extract were soluble in all the solvents used, whereas aqueous extract were soluble only in dimethyl sulfoxide and water. The observations of physiochemical were depicted in (**Table 1)**.

Table 1: Organoleptic and Physiochemical analysis of G. asiatica leaves and extracts.

Physiochemical			
Properties	**Dried Powder**	**Methanol Extract**	**Aqueous Extract**
Appearance	Dried	Semi-Dried	Semi-Dried
Taste	Slightly Bitter	Bitter	Bitter
Odor	Aromatic	Sweet	Sweet
Color	Greenish- Brown	Dark Brown	Dark Brown
Extractive Yield	-	15.335gm	33.238gm
pH	-	5.42	6.34
Solubility			
Solvents		**Methanolic Extract**	**Aqueous Extract**
Petroleum Ether		Freely Soluble	Insoluble
Ethanol		Soluble	Insoluble
Ethyl Acetate		Soluble	Insoluble
Dimethyl Sulfoxide		Soluble	Partially Soluble
Water		Partially Soluble	Freely Soluble
Methanol		Soluble	Insoluble
Chloroform		Soluble	Insoluble
Acetone		Freely Soluble	Insoluble

3.2 Qualitative Analysis of Phytoconstituents

The secondary phytochemicals have possible pharmacological properties, such as antidiabetic, antitumor, antimicrobial and anti-inflammatory **[24]**. The extracts of G. asiatica were investigated for phytochemical analysis, which revealed the existence of secondary phytochemicals, **Table 2**. Preliminary investigations verified the existence of triterpenoids, sterol, flavonoids, protein, carbohydrates and alkaloid and absence of tannins in both the extracts **[25]**. These findings

corresponds with the findings of previous researches **[26]**.

Table 2: Qualitative phytochemical screening of G. asiatica leaves extracts.

Qualitative screening (P = present, A= absent)			
Method		**Methanolic**	**Aqueous**
Carbohydrates	Molisch's	P	P
	Fehling's	P	P
	Barfoed's	P	P
Alkaloid	Mayer's	P	P
	Dragendroff's	P	P
	Tannic	P	P
Terpenoids	Salkowski	P	P
	Libermann-Burchard's	P	P
Steroids	Salkowski	P	P
	Libermann-Burchard's	P	P
Flavonoids	Zn. Hydrochloride	P	P
	Lead Acetate	P	P
	Shinoda	P	A
Phenols	Iodine	P	P
	Gelatine	P	P
	Lead Acetate	P	P
Tannins	Lead sub acetate	A	A
	Sodium hydroxide	A	A
Protein	Biuret's	P	P
	Ninhydrin	P	P
Saponins	Froth	P	A
Glycosides	Legal's	P	P
	Baljet's	P	A
	Borntrager's	P	A

3.3 Quantification of TPC

Phenolic substances play a vital role in cellular architecture and function as integral precursors to polymeric phenols **[27]**. The TPC was calculated by calibration curve of gallic acid (GA) and the TPC was decoded as mg/100 mg of GA equivalent to that of the dried sample and is shown in **Figure 1 (Table 3)**. Among the two crude extracts, methanolic extract contained the highest (48.82 mg GAE/gm dried extract) amount of phenolic compounds followed by aqueous extract (38.34 mg GAE/gm dried extract).

3.4 Quantification of TFC

Flavonoids form stable complexes with aluminium chloride via their carbonyl (C4) and hydroxyl (C3 of flavonols and C5 of flavonols). Additionally, ortho hydroxyl group on B ring lead to formation of unstable acidic complexes [28]**.** The TFC was estimated by standard calibration curve of rutin (R) and the content is mentioned as mg/ 100mg of rutin equivalent (RE) relative to the dry sample (**Figure 1, Table 3)**. Among both crude extracts, methanolic extract contains highest (165.23 mg RE/g dried extract) amount of flavonoid followed by aqueous extract (58.16 mg RE/g dried extract).

3.5 Quantification of TAC

TAC was estimated by calibration curve of atropine and the content is mentioned as mg/100 mg of atropine equivalent (AE) relative to the dry sample, as shown in **Figure 1 (Table 3)**. Among both extracts, aqueous extract contained the highest (56.107 mg AE/g) amount of alkaloid compounds followed by methanolic extract (20.73 mg AE/g).

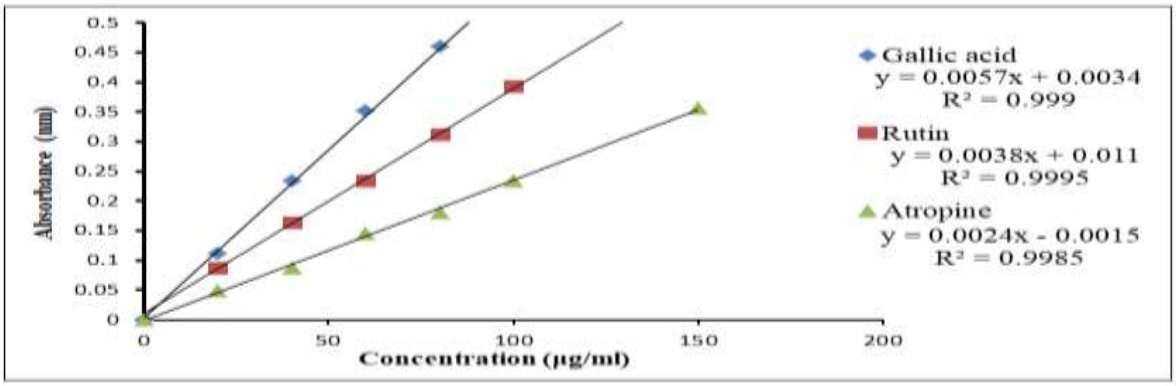


Figure 1: Calibration curve of TPC, TFC and TAC.

Table 3: Quantitative phytochemical screening of G. asiatica leaves extracts.

Extract	TPC	TFC	TAC
Methanolic	48.82 mg GAE/gm	165.23mg RE/g	20.73 mg AE/g
Aqueous	38.34 mg GAE/gm	58.16 mg RE/g	56.107 mg AE/g

3.6 Thin-Layer Chromatography

TLC is preferred for the quantitative estimation of chemical blends (on the basis of the binding affinity of the chemical and mobile phases on silica), supports characterization homogeneity and is an established criterion for validation **[29]**. TLC was assayed to investigate the chemicals of methanolic extract of the leaves. TLC in Toluene:ethyl acetate:acetic acid (6:4:0.8) solvent system clearly revealed visible bands. The color and Rf values of the different bands and standard Nuciferine under UV light are depicted in **Table 4, Figure 2**. TLC confirmed presence of nine different phytochemicals and absence of Nuciferine in methanolic extract. Recorded Rf values of mostly spots were >0.5, revealing polar phytochemicals **[17]**.

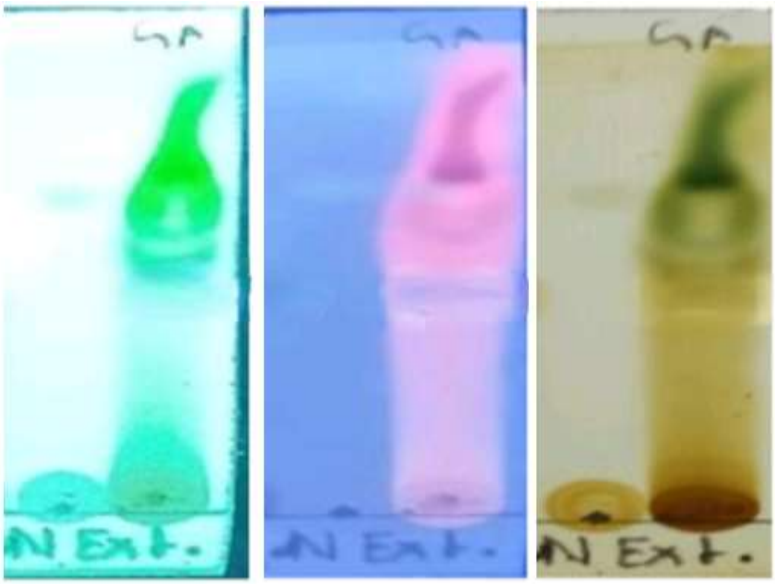


Figure 2: TLC estimation of methanolic extract and Nuciferine (N) by UV lamp.

Table 4: TLC of methanolic extract of G. asiatica leaves (L=light, D= dark).

S. no.	Sample	Colour at 254 nm	Colour at 365 nm	Rf
1.	Methanolic extract	Green	L. Pink	0.28
2.		L. green	Florescence	0.50
3.		Green	L. Blue	0.56
4.		D. green	L. Pink	0.64
5.		D. green	Pink	0.66
6.		Green	Florescence	0.70
7.		D. green	D. pink	0.76
8.		L. Green	Yellow	0.82
9.		L. green	Pink	0.96
10.	Nuciferine	-	-	-

3.7 Determination of antioxidant activity

The impact of methanolic extract on the levels of LPO, GSH and SOD is depicted in **Table 5, Figure 3**. Positive control Group animals showed significant reduction in SOD (116.88±0.20 U/L) and GSH (42.34±2.051 nmol/g) while, increase in LPO (46.676±0.836 nmol/g), Compared to normal control group SOD (170.35±0.31 U/L), GSH (82.75±3.121 nmol/g) and LPO (8.172±0.1189 nmol/g), respectively **[30]**. The results of SOD and GSH in positive control group coincides with the previous studies **[31][32]**. Treatment in group IV, V and VI significantly increase SOD (143.7±0.74, 155.13±0.24 and 161.0±0.158 U/L) and GSH (45.74±0.122, 54.82±2.095 and 64.03±1.040 nmol/g) while, decrease in LPO (42.216±0.976, 23.856±0.849 and 8.958±0.054 nmol/g) compared to positive control group. Silymarin in standard group significantly increase SOD (167.41±0.44 U/L) and GSH (76.33±4.118 nmol/g) while, decrease in tissue LPO (7.158±0.088 nmol/g tissue) compared to positive control group. These indicated that methanolic extract of plant reduce oxidative radicles with inflammation in a dose gradient pattern. Several studies documented radicle quenching potential of phytochemicals, majorly phenolic and flavonoids **[33][34]**. Hence, might be the potent effect against carbontetrachloride induce oxidative stress due to presence of bioactive phenols and flavonoids.

Table 5: **Impacts of methanolic leaves extract of G. asiatica on in-vivo Antioxidant activity.**

GROUPS	SOD (U/L)	GSH (nmol/g)	LPO (nmol/mg)
Normal Control group	170.35±0.31	82.75±3.121	8.172 ±0.1189
Positive control group	116.88±0.20$^{\#}$	42.34±2.051$^{\#}$	46.676 ±0.836$^{\#}$
Standard group	167.41±0.44****	76.33±4.118****	7.158 ±0.088****
Treatment group I	143.7±0.74***	45.74±0.122	42.216 ±0.97***
Treatment group II	155.13±0.24***	54.82±2.095**	23.856 ±0.849****
Treatment group III	161.0±0.158****	64.03±1.04****	8.958 ±0.054****

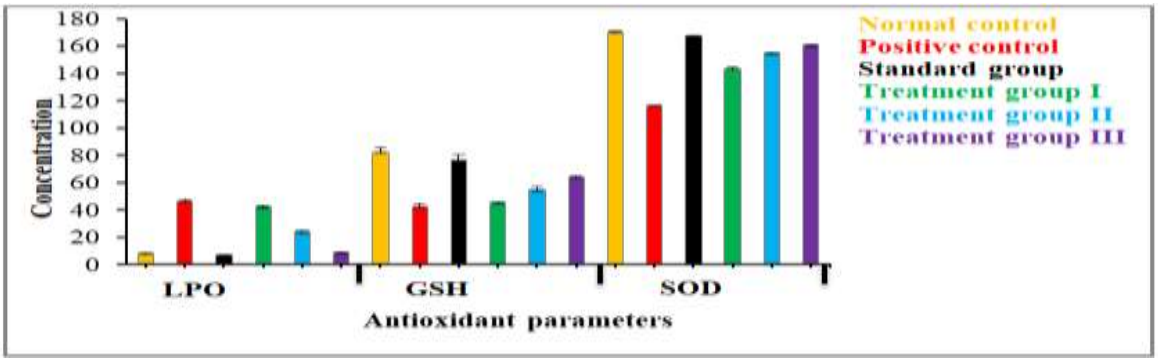


Figure 3: Impacts of methanolic extract on Antioxidant activity.

4. CONCLUSION

In current study, it was demonstrated that the majority of bioactive constituents were present in both methanolic and aqueous extracts; however, the methanolic extract contained a range of phytochemicals. The bioactive phytochemicals are suitable substitutes for oxidative stress reduction. Administration of methanolic extract produce significant radical scavenging activity in SOD, GSH and LPO analysis against carbontetrachloride induced oxidative stress in rats. The proclaimed impact was at higher dose level, and reported bioactive compounds of the leaves, possibly be responsible for this. Thus, hinting that Grewia asiatica might capable for controlling different oxidative stress induced diseases. However, other pharmacological activity, isolation, purification, and structural characterization of bioactive phytocompounds are needed to be further explored the therapeutic effect of the plant in the future. These systematic points of view are likely to provide a greater mechanistic understanding, help in the development of pharmaceutical formulation to strengthen its remedial benefit.

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7. FUNDING: None
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