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Extraction Techniques Optimization, Suitable Solvents Selection and *In Vitro* Evaluation of Lipid Peroxidation Inhibition of Five Traditional Medicinal Plants using Egg Yolk, Rat Liver and Rat Brain Homogenates

Sutar Ravindra Chandrakant[1], Sohi Shaheena[2], Mansoor Mohammad[3], Singh Dharmendra[4], Satapathy Ashok Kumar[5], Singh Kiran[6], Kumar Satendra[7], Bhaskar Roushan[8] and Bohra Dau Lal[9]*

[1]Department of Pharmacology, SRES'S Sanjivani College of Pharmaceutical Education and Research (Autonomous), Kopargaon , Ahmednagar, Maharashtra, India
[2]College of Pharmacy, RIMT University, Mandi Gobindgarh, Fatehgarh Sahib 147301, Punjab, India
[3]Department of Pharmacology, Victoria Vollege of Pharmacy, Nallapadu, Guntur, Andhra Pradesh, India
[4]Institute of Pharmacy, Veer Bahadur Singh Purvanchal University, Jaunpur 222003, Uttar Pradesh, India
[5]Acharya Institute of Technology, Soldevanahalli, Hesaraghatta Main Road, Bangalore, Karnataka, India
[6]Nirmala Devi Pharmacy College, Gaurabadshahpur, Jaunpur, Uttar Pradesh, India
[7]Principal of L.N.Pharmacy College Baitalpur Deoria 274201, Uttar Pradesh, India
[8]M.Pharm (Pharmacology), St. Soldier Institute of Pharmacy, Jalandhar, India
[9]Department of Zoology, Seth GB Podar College, Nawalgarh, Jhunjhunu, Rajasthan, India

**Corresponding Author*

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Abstract: The present study aimed to optimize a solvent selection and extraction method selection process for extraction of a few traditional medicinal plants. The ability of the medicinal plant extracts to inhibit lipid peroxidation process had also been investigated in this study. Based upon previous studies and traditional literature review, five traditional medicinal plants have been selected for extraction and further study. These five traditional plants included *Paederia foetida, Alternanthera sessilis, Prunella vulgaris, Solidago virga-aurea Linn., and Rumex acetosella.* Pilot level extraction has been performed with several solvents based on polarity and phytochemical screening has been performed to select the best suitable solvent for extraction for each plant. Similarly, three methods of extraction viz. cold maceration, Soxhlet extraction and microwave assisted extraction methods have been used for extraction purpose. For each plant optimized the best extraction method based upon phytochemical screening. Hereafter, with a suitable solvent and a best extraction method as optimized, extraction of the five medicinal plants has been done and subjected to lipid peroxidation inhibition assay in three *in vitro* models namely, egg yolk homogenate, rat liver homogenate and rat brain homogenate models. The findings of the study demonstrated optimization of the selection of solvent as well as extraction method and further exhibited significant lipid peroxidation inhibition by all the studied extract. However, *Paederia foetida*, in particular, was found to possess the highest and consistent level of LPO inhibition, showing it has strong antioxidant properties.

Keywords: Extraction methods, Solvent selection, *In vitro* evaluation, Lipid peroxidation inhibition, *Paederia foetida, Alternanthera sessilis, Prunella vulgaris, Solidago virga-aurea* Linn., *Rumex acetosella*

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Introduction

The buildup of lipid peroxidation (LPO) products in human tissues is a primary factor in cellular and tissue dysfunction, which is linked to ageing and the majority of illnesses associated with oxidative stress. The available data support the involvement of LPO in pathogenic processes. A review of the literature and growing body of data are indicating the function of LPO in the pathophysiology of ageing and illnesses that are traditionally connected to oxidative stress, such as diabetes, atherosclerosis, and neurodegenerative disorders. The published literature produced many compelling evidence linking folic acid production (LPO) to foetal vascular dysfunction in pre-eclampsia, liver and kidney disorders, and as a cause and consequence of cancer development (Luper, 1999; Khoshakhlagh *et al*., 2023; Mendoza-Fernández *et al*., 2023). Lipids including unsaturated fatty acids with many double bonds are more vulnerable to the effects of free radicals when it comes to biological molecules. Lipid peroxidation, the ensuing process, severely damages cellular membranes' structure and function by disrupting them. Numerous studies have been conducted on lipid peroxidation in connection to illness, antioxidant regulation, and other situations. During this procedure, a lot of byproducts are produced. Various assays can be used to measure these. The most widely used technique involves estimating aldehydic compounds based on their capacity to react with thiobarbituric acid (TBA) to produce "thiobarbituric acid reactive substances" (TBARS), which can be detected spectrophotometry with ease (Tung *et al*., 2019).

Reactive oxygen species (ROS) are produced by a variety of metabolic processes in our bodies. These reactions primarily produce free radicals and excited states, which have the potential to harm important biomolecules. Oxidative stress occurs if the antioxidant defense mechanism in the tissues is unable to neutralize them adequately (Luper, 1999; Khoshakhlagh *et al*., 2023; Mendoza-Fernández *et al*., 2023). When it comes to the biology of oxidative stress brought on by ROS, polyunsaturated fatty acids have emerged as a key player. These fatty acids are mostly found in the phospholipids of biological membranes, and the unspecific oxidation of these fatty acids by reactive oxygen species (ROS) is a very damaging process that is mediated by ROS. This process is known as lipid peroxidation. It produces harmful byproducts including 4-hydroxy-nonanal, which is known to change other biomolecules within the cell (Luper, 1999; Khoshakhlagh *et al*., 2023; Mendoza-Fernández *et al*., 2023; Saha *et al*., 2023).

A wide range of antioxidant defence systems exist in cells to either minimise the harmful effects of free radicals or prevent their production (Kirtikar *et al*., 1935; Jangam *et al*., 2024) The body needs antioxidant supplements to lessen oxidative damage and delay lipid peroxidation since these processes are insufficient when the balance tips in favour of free radical formation. The intrinsic toxicity of synthetic antioxidants at optimal concentrations has restricted their usage in modern times. There is a lot of interest in the application of natural antioxidants derived from plants. Plant phytochemical components have been shown to be lipid peroxidation inhibitors and free radical scavengers (Luper, 1999; Tung *et al*., 1999; Jangam *et al*., 2024).

Associating oxidative damage with inflammation, neurological and cardiovascular

diseases, measuring inhibition in controlled settings provides insights into plants' potential protective, antioxidant qualities. Numerous bioactive compounds - alkaloids, polyphenols, flavonoids, sterols, glycosides, terpenoids can neutralize free radicals, hinder lipid peroxidation. Assays assess extracts' abilities to counteract, neutralize oxidative stress, a factor in many disorders. This method furthers understanding of plants' protective, health contributions (Singh *et al*., 2018; Steven *et al*., 2019; Sharma *et al*., 2021; Mohyeldin *et al*., 2023).

We can measure how well some traditional medicinal plants prevent lipid peroxidation. This process happens in tissues like the- brain, liver, and egg yolks. After screening plants, we found a few that limit peroxidation significantly. For example, *Rumex acetosella, Solidago virga-aurea* Linn., and *Paederia foetida* strongly inhibited peroxidation across tissues. Testing extracts in varied biological systems shows their broad effects. Understanding how traditional plants impact different living tissues reveals their potential benefits. Extracts that widely block peroxidation may have diverse biological activity (Thabrew *et al*., 1997; Tung *et al*., 1999; Singh *et al*., 2018; Steven *et al*., 2019; Sharma *et al*., 2021; Khoshakhlagh *et al*., 2023; Mohyeldin *et al*., 2023; Saliani *et al*., 2023).

To evaluate a plant extract's skill to inhibit lipid peroxidation, we compare it against standards like quercetin and catechin. These famous antioxidants readily reduce lipid peroxidation. Adding these standards to the analysis reveals how well plant extracts inhibit lipid peroxidation. These assays unveil extracts' protective, antioxidant properties, evaluating potential therapies. Combining traditional knowledge and modern science is key to expanding herbal therapy. This integration breeds comprehension of traditional medicinal plants' health and wellbeing benefits (Luper, 1999; Steven *et al*., 2019; Maleki *et al*., 2019). The present study thereby aimed to investigate five traditional medicinal plants viz., *Alternanthera sessilis*, *Paederia foetida*, *Rumex acetosella, Solidago virga-aurea* Linn., and *Prunella vulgaris.* These five medicinal plants have been selected based on published as well as traditional literatures (Warrier, 1993; Luper, 1999; Khare, 2008; Van de Venter *et al*., 2008; Palipoch *et al*., 2013; Zieneldien *et al*., 2022). This research also aimed to bridge the gap between traditional medicinal knowledge and modern scientific research. The present study also aimed to optimize the extraction method and evaluation of the five traditional medicinal plants including *Paederia foetida*, *Alternanthera sessilis*, *Prunella vulgaris*, *Solidago virga-aurea* Linn., and *Rumex acetosella* for lipid peroxidation inhibition and its role in oxidative stress in a mechanistic approach.

Materials and Methods

Plant Material and Authentication:

All of the plants were gathered from previously designated locations or from Global Herbs, located in Khari Bhaoli, New Delhi, India and Herbal Team from Himachal Pradesh, authorized and pre-validated herbal crude drug suppliers. Every plant sample was identified botanically.

Pilot level extraction: selection of a suitable solvent and extraction method:

Numerous solvents are available for the extraction of medicinal plants; nevertheless, the choice of an appropriate solvent capable of extracting the active ingredients is contingent upon both the active ingredients' chemical characteristics and the solvent's quality. In addition to having different polarity and boiling points, solvents can react differently with other compounds that they come into contact with. From this point forward, choosing an appropriate solvent was the first step in the successful extraction of medicinal herbs (Sasidharan *et al*., 2011).

Treatment of Plant Material and Selection of a suitable solvent:

After being shade-dried, the plant materials were ground into a powder using an electric grinder. In a test tube, 1 g of the crude drug's powder was dissolved in 10 ml of various solvents. The

solvents range in polarity from low to high. Ethanol, HPLC grade water, aqueous methanol, aqueous ethanol, ethyl acetate, chloroform, and acetone were the solvents used to extract the plants. For a day the test tubes were sealed with aluminium foil, and then the next day the test tubes were filtered. The filtrate was subjected to preliminary phytochemical tests. The results of the phytochemical test were utilized to determine the appropriate solvent for further extractions.

Phytochemical Investigation of pilot level extracts:

A range of tests uncovered the chemical makeup of the pilot extracts (Warrier, 1993) Screening checked for alkaloids, flavonoids, glycosides, saponins, phenols, phytosterols, terpenoids, carbohydrates, and proteins in the extracts. Further assessment provided additional details on these components present in the prepared samples.

Selection of a suitable extraction method:

(i) *Soxhlet extraction technique*:

In order to fully extract the phytochemicals from coarsely ground materials, the Soxhlet apparatus was used. Petroleum ether, chloroform, ethyl acetate, methanol, and water were used as solvents. The various extracts were dried at 45 °C in a rotary evaporator, and the dried extracts were then kept in vacuum desiccators with anhydrous silica gel within.

(ii) *Cold maceration technique*:

The cold maceration process was employed to extract the plants. For a duration of seven days, the dry drug powder was macerated in 80% methanol at room temperature with sporadic shaking and stirring. Following that, filtering was used to obtain the filtrate, which was then concentrated by reducing it in a water bath. To get the final extract, the extract was concentrated over a water bath (Sasidharan *et al*., 2011).

(iii) *Microwave assisted technique*:

The plants were extracted using a microwave-assisted process. Five grams of dry powder were dissolved in 100 ml of 90% methanol, and the microwave process was run at 160 W. After 5 min of the process, the temperature was allowed to drop to room temperature. The microwave was then turned on for a further minute. To obtain the final extract, the extract was filtered and then concentrated on a water bath (Mandal *et al*., 2007).

Phytochemical Screening of the extracts:

As previously mentioned, three distinct extraction techniques were used to produce the extracts. To check for the presence of alkaloids, flavanoids, glycosides, saponins, phenols, phytosterols, carbohydrates, and proteins, a preliminary phyto-chemical screening was performed on all the extracts. An extraction procedure was chosen for the final extract's further production based on the phytochemical screening results.

Mass extraction and Extractive yield:

One of the three techniques - cold maceration, microwave assisted, or Soxhlet extraction - was applied with the appropriate solvents for the mass extraction of each plant or plant part (Table 1) (Mandal *et al*., 2007; Sasidharan *et al*., 2011). The yield of the extracts made using different extraction methods was determined and presented in the Results section.

Qualitative phytochemical analysis:

A preliminary phytochemical screening was conducted on the extracts produced by the various extraction techniques to check for the presence of proteins, carbohydrates, phytosterols, alkaloids, flavanoids, glycosides, saponins, phenols, and phytosterols (Harborne, 1998).

In vitro LPO inhibition using egg yolk, rat liver and rat brain homogenates:

Using egg yolk, rat liver, and brain homogenates as lipid-fortified medium, an enhanced thiobarbituric acid reactive species (TBARS) assay was employed to quantify the lipid peroxide generated, as reported by (Ruberto *et al*., 2000). Thiobarbituric acid reactive substances are formed when lipids undergo peroxidation (TBARS). We quantify this

Table 1: Representing the plant part used, extraction method and the suitable solvent

Plant Name	Parts used	Solvent selected	Extraction method Selected
Rumex acetosella	Leaves	90 % methanol	Microwave assisted
Solidago virga-aurea Linn.	Whole plant except roots	80 % ethanol	Cold maceration
Paederia foetida	Whole plant	Methanol	Cold maceration/ Soxhlet extraction
Alternanthera sessilis	Leaves	Methanol	Cold maceration
Prunella vulgaris	Stem and leavces	Water/Methanol	Soxhlet extraction

Table 2: The yield of the extracts obtained

Medicinal Plant Names		Extractive Weight in grams and Values in Percentage
Rumex acetosella	Extractive weight	185.67 g
(1 kg)	Extractive value	18.57 %
Solidago virga-aurea Linn.	Extractive weight	117.27 g
(1 kg)	Extractive value	11.73 %
Paederia foetida	Extractive weight	79.45 g
(1 kg)	Extractive value	7.95 %
Alternanthera sessilis	Extractive weight	98.92 g
(1 kg)	Extractive value	9.89 %
Prunella vulgaris	Extractive weight	194.87 g
(1 kg)	Extractive value	19.49

using thiobarbituric acid (TBA), which combines with TBARS to generate a complex. By measuring the absorbance of this complex at 532 nm in a spectrophotometer the extent of lipid peroxidation can be estimated. Higher absorbance levels indicate more lipid peroxidation because they indicate the presence of more TBARS. We may see that treated samples have fewer TBARS by comparing those treated with antioxidants to those not treated in comparison to an untreated group. This implies that antioxidants work well to prevent lipid peroxidation.

Results and Discussion

Selection of a Suitable Solvent and Extraction Method:

Picking the right solvent and method for extracting phytochemical from medicinal plants is actually vital. It aids in the extraction of most of the phytochemicals from the plants. In this study, five different plants- *Paederia foetida, Alternanthera sessilis, Prunella vulgaris, Solidago virga-aurea* Linn., and *Rumex acetosella*, were extracted. The choice of solvent and method is a significant modality because it affects how well the phytochemicals from these plants get extracted.

Solvent Selection and Extraction Method Selection:

The best suitable solvent for extraction as well as suitable method for extraction for each plant have been evaluated and presented in Table 1.

Extractive yield and Qualitative phytochemical analysis:

Various solvents have been utilized for extraction along with several extraction methods including Soxhlet extraction, microwave-assisted extraction, and cold maceration techniques. The per centage yield for each extract and the best suitable method for extraction for each plant have been stated and outlined in Table 2.

The results for the preliminary phytochemical

Table 3: Preliminary phytochemical screening of the extracts

Phytoconstituents	**Qualitative Findings**				
	Rumex acetosella	*Solidago virga-aurea* Linn.	*Paederia foetida*	*Alternanthera sessilis*	*Prunella vulgaris*
Saponins	√	√	√	√	√
Sterols	√	-	√	√	√
Carbohydrates	√	√	√	√	√
Tannins	√	√	√	-	√
Flavanoids	√	√	√	√	√
Fatty acid	-	√	√	√	-
Terpenoid	√	√	√	√	√
Glycosides	√	-	√	√	-

'√'= Present and '-' = Absent

Table 4: Result for *in vitro* Lipid peroxidation Inhibition (LPO) in egg yolk system

Plant NAME	**Extract Name**	**% Inhibition of LPO**
Rumex acetosella	RAME-90	74.93±0.995
Solidago virga-aurea Linn.	SVAEX-80	76.69±0.935
Paederia foetida	PFME	78.66±1.336**
Alternanthera sessilis	ASME	63.88±1.744
Prunella vulgaris	PVWME	69.73±0.898
Standard 1	Quercetin	89.61±1.079
Standard 2	Catechin	93.35±1.912

**$p < 0.01$

screening have been tabulated and presented in Table 3. All the tested major phytochemicals were found to be present in all the five extracts. However, fatty acid was found to be absent in *Rumex acetosella* extract, glycosides is absent in *Solidago virga-aurea* Linn. extract, tannins is absent in *Alternanthera sessilis* extract, and fatty acid and glycosides were not present in *Prunella vulgaris* extract (Table 3) (Harborne, 1998).

Inhibition of lipid peroxidation using rat liver, rat brain and egg yolk homogenates:

A modified thiobarbituric acid reactive species (TBARS) method was employed to quantify the lipid peroxide generated, as previously mentioned (Chopra *et al*., 1956; Badmus *et al*., 2011). The homogenates of rat liver, rat brain and egg yolk had been used as a lipid-rich media. Lipid peroxidation inhibition experiment was performed on 100 µg/ml of each extract solution (Table 4). *Rumex acetosella, Solidago virga-aurea* Linn., *Paederia foetida, Alternanthera sessilis*, and *Prunella vulgaris* are the five medicinal plants whose extracts were evaluated *in vitro* for their possible antioxidant capabilities utilizing homogenates of egg yolk, rat liver, and rat brain. For every plant extract, the % inhibition of LPO was determined, and the outcomes were contrasted with quercetin and catechin, two common antioxidants.

Egg Yolk LPO Inhibition:

All plant extracts demonstrated notable inhibition of lipid peroxidation in the egg yolk experiment. With a per centage of 78.66, *Paederia foetida* (PFME) showed the highest level of inhibition, closely followed by *Solidago virga-aurea* Linn. (SVAEX-80) at 76.69, *Rumex acetosella* (RAME-90) at 74.93, *Prunella vulgaris* (PVWME) at 69.73, and *Alternanthera sessilis* (ASME) at 63.88. These findings imply that the extracts include bioactive substances that can stop or reduce lipid oxidative

Table 5: Result for *in vitro* Lipid peroxidation Inhibition (LPO) in rat liver homogenate.

Plant NAME	Extract Name	% Inhibition of LPO
Rumex acetosella	RAME-90	70.91±0.984
Solidago virga-aurea Linn.	SVAEX-80	72.75±0.926
Paederia foetida	PFME	74.86±1.349**
Alternanthera sessilis	ASME	58.91±1.49
Prunella vulgaris	PVWME	65.84±0.952
Standard 1	Quercetin	89.61±1.079
Standard 2	Catechin	93.35±1.912

**p < 0.01

Table 6: Result for *in vitro* Lipid peroxidation Inhibition (LPO) in rat brain homogenate

Plant NAME	Extract Name	% Inhibition of LPO
Rumex acetosella	RAME-90	73.87±0.983
Solidago virga-aurea Linn.	SVAEX-80	75.93±0.928
Paederia foetida	PFME	75.59±1.612**
Alternanthera sessilis	ASME	69.74±1.873
Prunella vulgaris	PVWME	64.68±0.979
Standard 1	Quercetin	89.61±1.079
Standard 2	Catechin	93.35±1.912

**p < 0.01

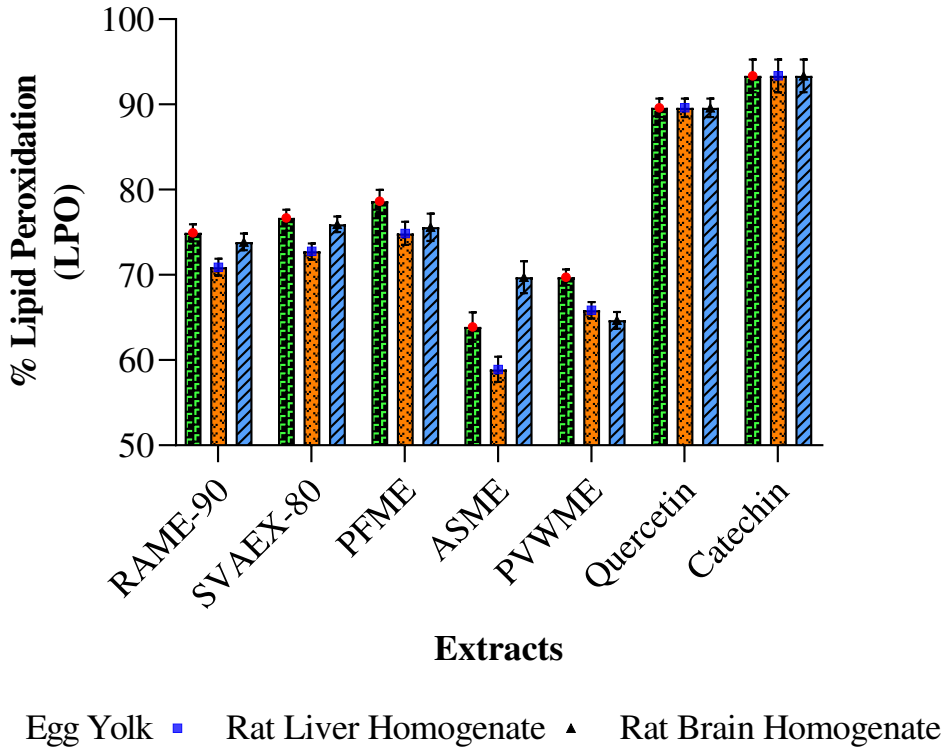

Fig. 1: A relative comparison of *in vitro* Lipid peroxidation (LPO) Inhibition of the extracts in different models.

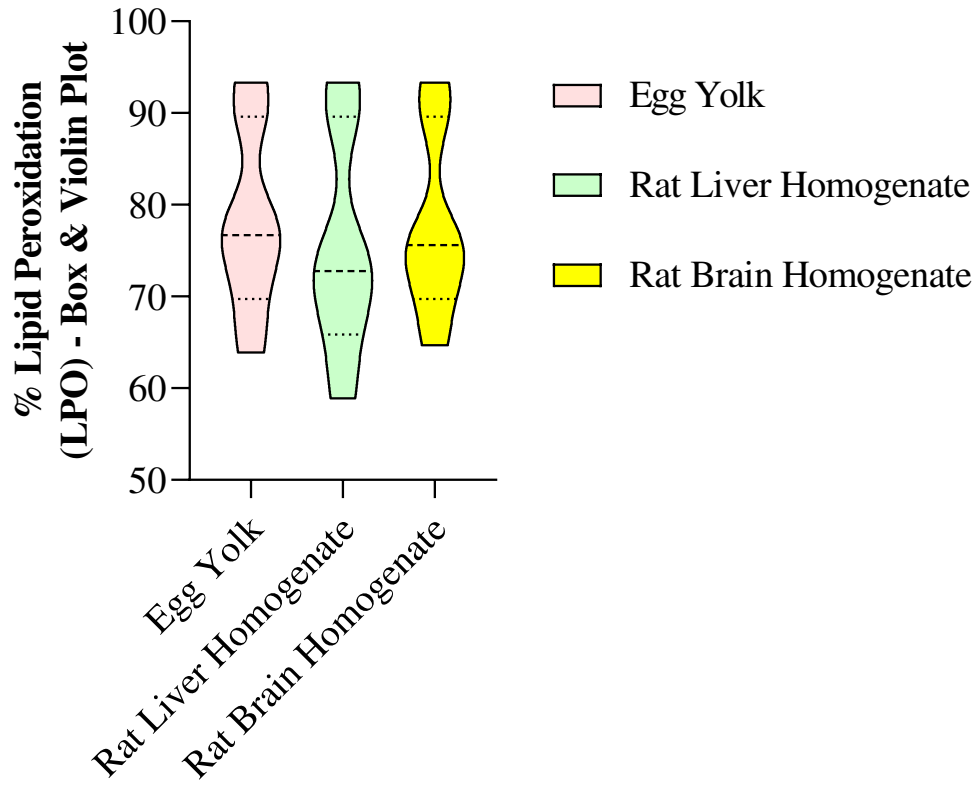

Fig. 2: A Box and Violin representation of Lipid peroxidation (LPO) Inhibition in vitro in various models.

breakdown (Table 4).

Rat Liver LPO Inhibition:

Similar findings were noted in the rat liver homogenate model as well. Once more, the most inhibited species was *Paederia foetida* (PFME) with 74.86 per cent. *Solidago virga-aurea* Linn. (SVAEX-80) came in second with 72.75 per cent, *Rumex acetosella* (RAME-90) with 70.91 per cent, *Prunella vulgaris* (PVWME) with 65.84 per cent, and *Alternanthera sessilis* (ASME) with 58.91 per cent (Fig. 1). These findings suggest that by reducing lipid peroxidation in the liver, plant extracts may have hepatoprotective qualities (Table 5).

Rat Brain LPO Inhibition:

Again, *Paederia foetida* (PFME) showed the highest inhibition (75.59 per cent) in the rat brain homogenate model. *Solidago virga-aurea* Linn. (SVAEX-80) showed the second-highest inhibition (75.93 per cent), followed by *Rumex acetosella* (RAME-90) at 73.87 per cent, *Prunella vulgaris* (PVWME) at 64.68 per cent, and *Alternanthera sessilis* (ASME) at 69.74 per cent (Fig. 2). By reducing oxidative stress caused by lipid peroxidation, these plant extracts may have neuroprotective potential, as indicated by the strong inhibitory effects seen in the brain model (Table 6).

Comparison with Standards:

It is clear from comparing the outcomes with quercetin and catechin standards that the plant extracts significantly suppress LPO. Despite the fact that the standards catechin in particular - exhibited stronger inhibitory effects, the plant extracts' performance is remarkable. Promising antioxidant activity is shown by the extracts' ability to approach or even surpass the well-established antioxidant criteria.

Overall Implications:

The findings of this study clearly demonstrated

antioxidant activity in terms of lipid peroxidation inhibition of all the studied five medicinal plant extracts. For each plant a suitable solvent and a suitable method for extraction had been researched and optimized. The lipid peroxidation inhibition denotes strong antioxidant capacity of the extract which in turn aid in fighting and neutralizing free radicals as well as augment the natural biological antioxidant defense system. *Paederia foetida*, in particular, was found to possess the highest and consistent level of LPO inhibition, showing it has strong antioxidant qualities. The presence of various classes of phytoconstituents like flavonoids, phenolics, sterols, glycosides, fatty acid and other antioxidant compounds might be the cause for the observed activities. Three different mechanisms such as non-enzymatic, enzymatic, free radical-mediated, and non-radical are used to oxidize lipids. Different compounds are produced by each oxidation pathway. The relative susceptibilities of lipids to oxidation depend on the reaction milieu as well as their inherent structure. Lipid hydroperoxides are formed as the major primary products, however, they are substrates for various enzymes and they also undergo various secondary reactions. Seleno-proteins, for instance, convert phospholipid hydroperoxides to their corresponding hydroxides *in vivo*. Therefore, it is very much necessary to inhibit all type of lipid peroxidation. Various types of antioxidants have different roles in preventing lipid peroxidation and the harmful consequences that result from the products of lipid peroxidation. In this study the plant extracts clearly demonstrated strong lipid peroxidation inhibition in all the three major lipid system, paving a way for further research.

Conclusion

In short, looking deeply into traditional medicinal plants, considering their history, culture, and science, shows how important they are in global healthcare. Plants like *Prunella vulgaris*, *Paederia foetida*, *Alternanthera sessilis*, *Rumex acetosella*, and *Solidago virga-aurea* Linn. have been traditionally mentioned in various literatures throughout the globe in many cultures. This study clearly demonstrated a optimization procedure for suitable solvent selection as well as suitable extraction method selection. Further all the five extracts from the medicinal plants significantly demonstrated lipid peroxidation inhibition. The results of this study paved a way for further study to evaluate the medicinal plants for *in vivo* studies in various animal disease models. The plants under discussion are examples of this convergence and provide insights into how traditional herbal medicine may be able to address modern health issues. The combination of evidence-based methods with conventional practices promises to lead to the development of sustainable, culturally appropriate, and successful healthcare solutions as long as this field's research is conducted.

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