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Research Article

PHYTOCHEMICAL CHARACTERIZATION AND ANTIOXIDANT ASSESSMENT OF HERBAL EXTRACTS

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ABSTRACT

Aim: The present study was aimed to evaluate and compare the antioxidant potential of the methanolic extracts *Plumbago zeylanica* Linn (PZME), *Datura stramonium* (DSME) and *Argemone mexicana* Linn (AMME) with its polyherbal methanol extract (PHME) composition.

Material and method: All the extracts were screened for the presence of various phytochemical by known and standard methods. Qualitative estimation of some potential phytoconstituents like flavonoids and polyphenolics was characterized by TLC (thin layer chromatography) and FTIR (Fourier Transform Infrared Spectroscopy). The quantification of the Rutin (flavonoid) and Gallic acid (polyphenol) were carried out using UV-visible spectrophotometry. The antioxidant activity of methanolic plant extracts and their combinations were assessed by DPPH (1, 1-diphenyl-2-picryl-hydrazil) method. Ascorbic acid was used as a standard and the antioxidant potential were evaluated at 2, 4, 6 and 8 mg/ml concentration.

Results: The phytochemical characterization of PZME, DSME and AMME revealed the presence of tannins, alkaloids, phenols and flavonoids. The polyherbal methanol extract (PHME) was found to exhibit potent antioxidant activity with maximum free radical scavenging activity (FRSA) of 82.79% as compared to standard ascorbic acid (92.92%) at a concentration of 8 mg/ml and was found to be significantly greater than individual methanol plant extracts.

Conclusion: The study revealed a potent synergistic antioxidant effect of methanol extracts of *Plumbago zeylanica* Linn, *Datura stramonium* Linn and *Argemone mexicana* Linn which can be successfully used to mitigate oxidative stress associated complications and other regenerative therapies.

Keywords: *Plumbago zeylanica* Linn, *Datura stramonium* Linn, *Argemone mexicana* Linn, Antioxidant, DPPH (1, 1-diphenyl-2-picryl-hydrazil), FTIR, Rutin, Gallic acid.

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INTRODUCTION

The oxidative environment presents a range of free radicals like superoxide, hydroxyl radical, nitric oxide and peroxyxynitrite, for interaction with living organisms. These are the evidences that these free radicals cause various diseases like Cancer, neuro-degeneration and some inflammatory disease¹⁻². It has been reported as an inverse relationship between antioxidative status and incidence of human diseases such as cancer, aging, neurodegenerative disease, and atherosclerosis³. Antioxidants have therefore gained importance in recent times for their capacity to neutralize free radicals. The plants produce significant number of antioxidants to

prevent the oxidative stress caused by photons and oxygen thus can be a potential source for newer compounds with antioxidant activity.

Argemone mexicana Linn (Family *Papaveraceae*) known as Ghamoyais an exotic weed indigenous in South America, but has widespread distribution in many tropical and sub-tropical countries including West Africa. It is considered as an important medicinal plant in India. The yellow juice, which exudes when the plant is injured, has found usage as traditional medicine in India for dropsy, jaundice, ophthalmia, scabies and cutaneous infections. Its various parts have been used in chronic skin diseases, emetic, expectorant, demulcent

and diuretic, while its seeds and seed oil have been used for the treatment of dysentery, ulcers, asthma and other intestinal infections⁴.

Datura stramonium Linn., a wild-growing plant belonging to the *Solanaceae* family, is widely distributed and easily accessible worldwide. It contains a variety of toxic tropane alkaloids such as atropine, hyoscamine, and scopolamine and has been used in Ayurveda for curing various ailments like ulcers, wounds, inflammation, rheumatism, gout, sciatica, bruises, swellings, fever, asthma, bronchitis and toothache. Previous studies have revealed pharmacological effects of this plant, however, complete information regarding its pharmacology, toxicity, ethnobotany and phytochemistry still remain⁵.

Plumbago zeylanica Linn. is commonly known as white chitraka, belonging to the family *plumbaginaceae*. It is distributed as a weed throughout the tropical and subtropical countries of the world. It is a perennial sub-scandent shrub, growing throughout India, especially in Bengal, Uttar Pradesh, Kerala, Andhra Pradesh, Karnataka and Sri Lanka in most places. Traditionally it is used as a stimulant, digestant, expectorant, laxative, abortifacient and also in the treatment of muscular pain and rheumatic disease⁶.

The usefulness of the pharmacologically active ingredients of the Ayurvedic medicines has been established. The phytochemical constituent in the herbals such as saponins, tannins, alkaloids, alkenyl phenols, flavonoids, terpenoids, phorbol esters and sesquiterpenes lactones have desired healing effects. A single herb containing more than one of the phytoconstituents, works synergistically producing the derived pharmacological action⁷.

The polyherbalism offers more benefits as compared to a single herbal formulation and the derived therapeutic effect can be achieved with a single multi-constituent formulation. The lower dose of such herbal preparations would lead to reduced risk of deleterious side effects and multi drug dosing. These benefits have escalated the popularity of polyherbalism in the market as compared to a single herbal formulation. Based on these assumptions the present study was under taken to investigate and compare the antioxidant potential of the methanol extracts of *Plumbago zeylanica* Linn, *Datura stramonium* Linn and *Argemone mexicana* Linn. with their polyherbal compositions.

MATERIAL AND METHOD

Plant collection and authentication

The stem, leaf and aerial parts of *Plumbago zeylanica* Linn, *Datura stramonium* Linn and *Argemone mexicana* Linn respectively, were collected in the month of November from Mohanlal Sukhadia University campus, Udaipur, Rajasthan, India. Plant materials were identified and authenticated by the Botanical Survey of India, Arid Zone Regional Centre, Ministry of Forest and Climate Change, Jodhpur, Rajasthan, India.

Extraction

The extraction was individually carried out for different selected plants. The plant materials were shade-dried for 2 weeks, pulverized to a coarse powder, and passed through a sieve 20 to maintain uniformity. The coarsely dried powder was first treated with petroleum ether (60–80 °C) for 24hrs to remove fatty materials and then re-extracted by methanol and using soxhlet apparatus at predefined temperatures⁸⁻¹⁰. The extract was collected, filtered through Whatman filter paper, and concentrated in vacuum under reduced pressure. The dried extracts (*Plumbago zeylanica* Linn methanol extract: PZME, *Datura stramonium* Linn methanol extract: DSME and *Argemone mexicana* Linn methanol extract: AMME) were stored at 4°C for further study.

Phytochemical testing

The individual (PZME, DSME and AMME) as well as polyherbal methanol extract (PHME) were subjected to various phytochemical screening tests for the identification of the phytoconstituents present by using standardized procedures¹¹.

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR (Bruker Spectrometer, Germany) analysis of the PZME, DSME and AMME were performed and the spectrum was recorded in the range of 4000-400 cm^{-1} at a resolution of 4 cm^{-1} .

Estimation of Gallic acid and Rutin Content

The Gallic acid and Rutin content were determined by a standardized method with some modifications¹².

A stock solution of Gallic acid (100 $\mu\text{g/ml}$) was prepared in 50% methanol from which dilutions of 5, 10, 15, 20, 25, 30 and 35 $\mu\text{g/ml}$ were prepared. The methanol test extract (1 gm) was dissolved in 100 ml of methanol (50%). The absorbance of the Gallic acid and test sample were measured by UV spectrophotometer (Schimadzu, Japan) at λ_{max} of 272 nm against distilled water as blank. Quantification of Gallic acid was done from a standard curve as percentage w/w Gallic acid content.

Rutin (10 mg) was dissolved in 10 ml of methanol (80%) and then serial dilution of 10, 20, 30, 40 and 50 mg/ml was prepared. About 100 mg of methanol extract was dissolved separately in 100 ml of methanol (80%). The absorbance of the sample was measured at 260 nm using UV spectrophotometer, taking distilled water as blank. Quantification was done on the basis of the standard curve of Rutin. Results were expressed as percentage w/w Rutin content.

Qualitative estimation of phytoconstituents by thin layer chromatography (TLC)

Thin layer chromatography was performed using standard methods (Harborne, 1998). Samples PZME, DSME and AMME (2 mg/ml) were dissolved in methanol. Solvent systems, developed by varying the concentration of mobile phases were used. The plates were visualized directly after drying and with the help of UV-TLC viewer (Figure 1). The R_f value of the different spots so obtained were calculated and these values were matched with the reference values¹³⁻¹⁵.

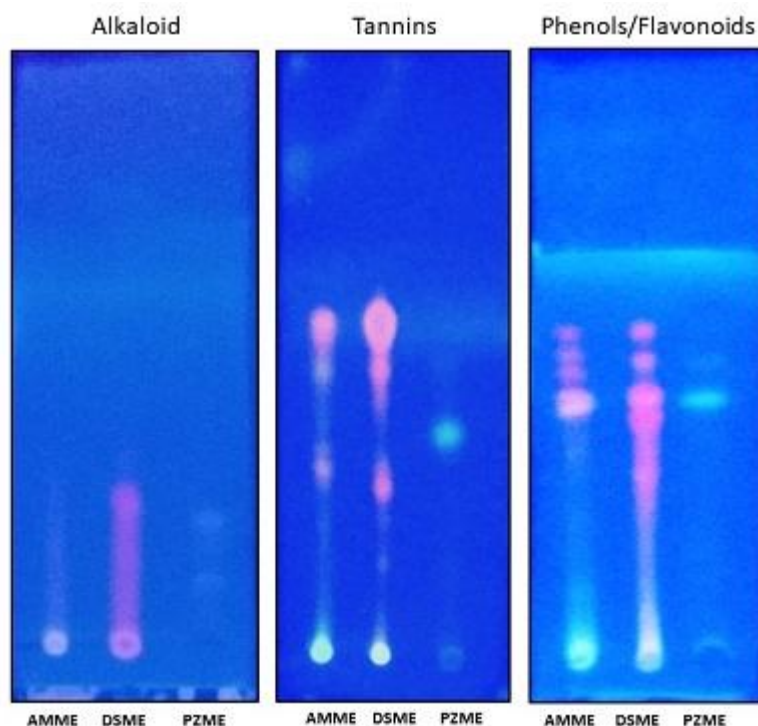


Figure 1: TLC of methanol extracts for alkaloids, tannins and phenolic/flavonoid compound characterization.

Development of Polyherbal Composition

The polyherbal formulation consists of combination of PZME, DSME and AMME. In case of phytochemical screening, equal proportions of the above extracts were taken, however the proportion of individual extract for polyherbal composition was decided on the basis of its individual *in-vitro* efficacy i.e. percentage free radical scavenging activity. The order of the antioxidant activity of methanol extracts was found to be in the order of PZME>AMME>DSME. Hence the proportion of the extracts was taken in the order of 5:3:2.

In-vitro antioxidant activity of poly herbal methanol extract (PHME) by DPPH method

The free radical scavenging activity of the individual extract as well as poly-herbal combination was measured by standardized 1, 1-diphenyl-2-picrylhydrazil (DPPH) method with some modifications¹⁶. DPPH solution (0.1 mM) was prepared in methanol. Higher concentrations of DPPH will show the absorbance beyond the accuracy of spectrophotometric measurement¹⁷⁻¹⁸, so the concentration of DPPH was so chosen such that it obeys Beer's Lambert laws. From the stock solution (10mg/ml) of ascorbic acid and methanol extract test samples (PZME, DSME, AMME and PHME), serial dilution of 2, 4, 6 and 8 mg/ml

concentration were prepared. To 50µl of test sample of different concentration, 1ml of DPPH solution (0.1 mM) was added in each test tube. The final volume in each test tube was made up to 3 ml with methanol. The reaction mixture was shaken vigorously and allowed to stand at room temperature for 30 minutes. Absorbance of the resulting mixture was measured at 517 nm against methanol as blank using a UV-visible spectrophotometer. Each sample was measured in triplicate and the results were represented as mean. The ascorbic acid was used as a standard antioxidant in this method. Percentage of DPPH free radical scavenging activity (FRSA) was determined as follows:

$$\% (FRSA) = \frac{(Absorbance\ of\ control - Absorbance\ of\ test\ sample)}{Absorbance\ of\ control} \times 100$$

The absorbance of DPPH was found to decrease by an exposure to light because of changes in its magnetic property so the procedures were carried out in a dark chamber (Figure 2). Standardized precautionary methods were adopted to prevent changes in the magnetic properties of the DPPH especially during the working and storage. A freshly prepared DPPH solution in methanol, stored under ambient laboratory temperature (24-25°C) in the dark was utilized within 24 hours¹⁹.

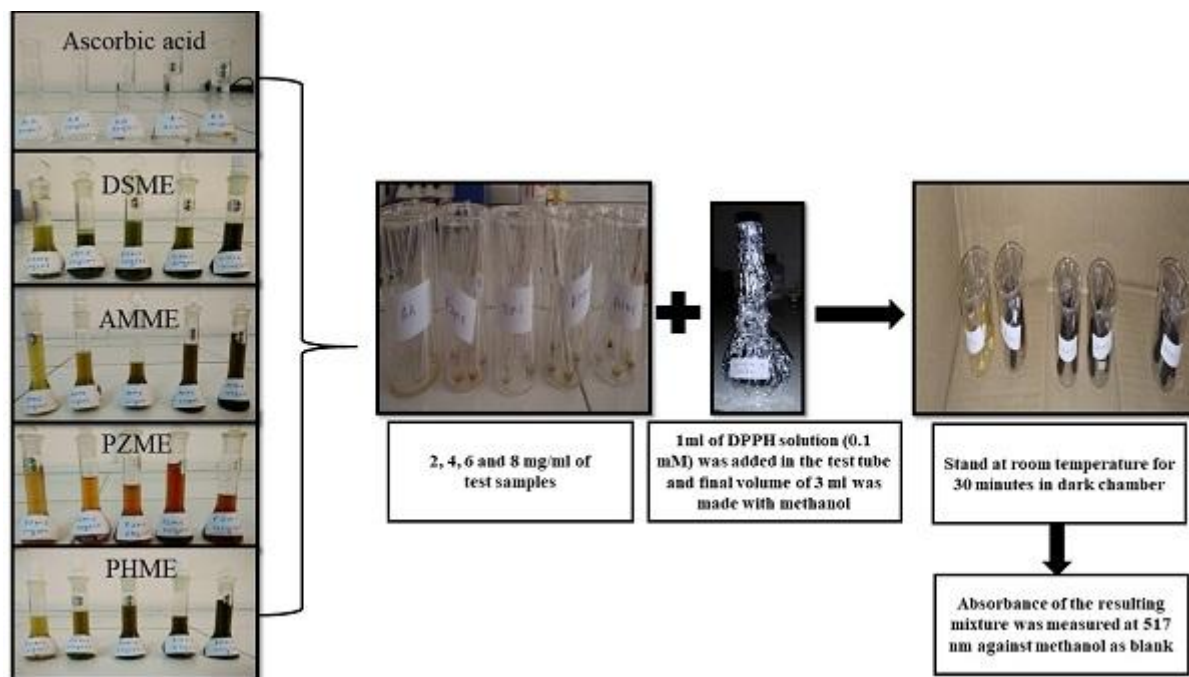


Figure 2: Scheme of *in-vitro* antioxidant DPPH method

RESULT

Phytochemical investigation of PZME, DSME and AMME revealed the presence of diverse phytoconstituents.

Table 1: Preliminary phytochemical screening of different methanol plant extracts

Phytoconstituents	Inference			
	PZME	DSME	AMME	PHME
Triterpenoids	+	+	+	+
Tannins	+	+	+	+
Steroids and sterols	+	+	+	+
Saponins	+	+	+	+
Proteins	+	+	+	+
Glycosides	+	+	+	+
Flavonoids	+	+	+	+
Carbohydrates	+	+	+	+
Alkaloids	+	+	+	+

Plus (+) = Present, Minus (-) = Absent

The phytochemical screening showed that these plants extract after being incorporated as polyherbal (PHME) does not have any impact on its phytochemical composition and was found to possess all phytoconstituents as shown by other methanol extract (Table 1).

FTIR analysis

FTIR analysis was carried out to characterize methanolic extract obtained from each plant as shown in figure-3,4 and 5. It has been observed that PZME, AMME and DSME showed a characteristic peak of the carbonyl absorption band at 1653.61cm^{-1} , 1603.51cm^{-1} and 1660.03cm^{-1} respectively which is assigned to aromatic ketonic carbonyl stretching which corresponds to the presence of flavonoids. Characteristics peaks at 3408.82cm^{-1} , 3143.23cm^{-1} and 3409.99cm^{-1} of PZME, AMME and DSME respectively are due to hydroxyl stretching of alcohols and phenols of the flavonoids.

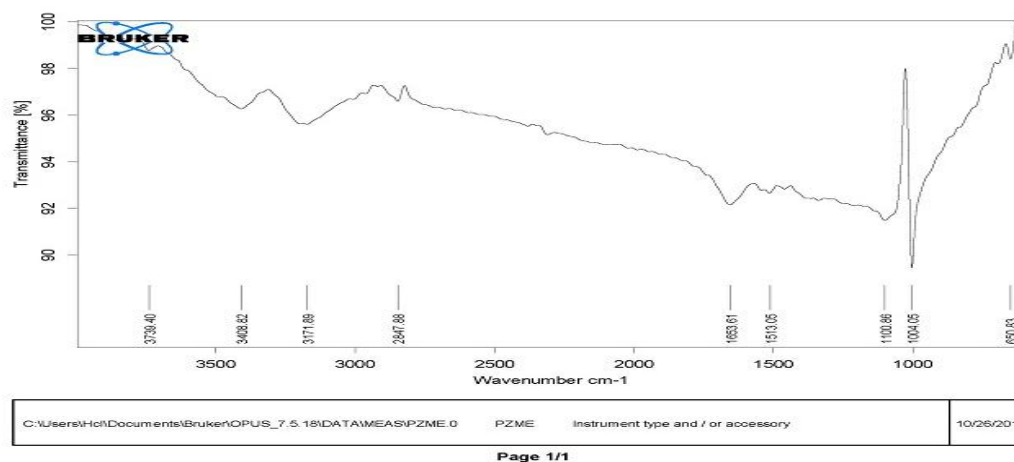


Figure 3: FTIR spectrum of *Plumbago zeylanica* Linn methanol extract (PZME)

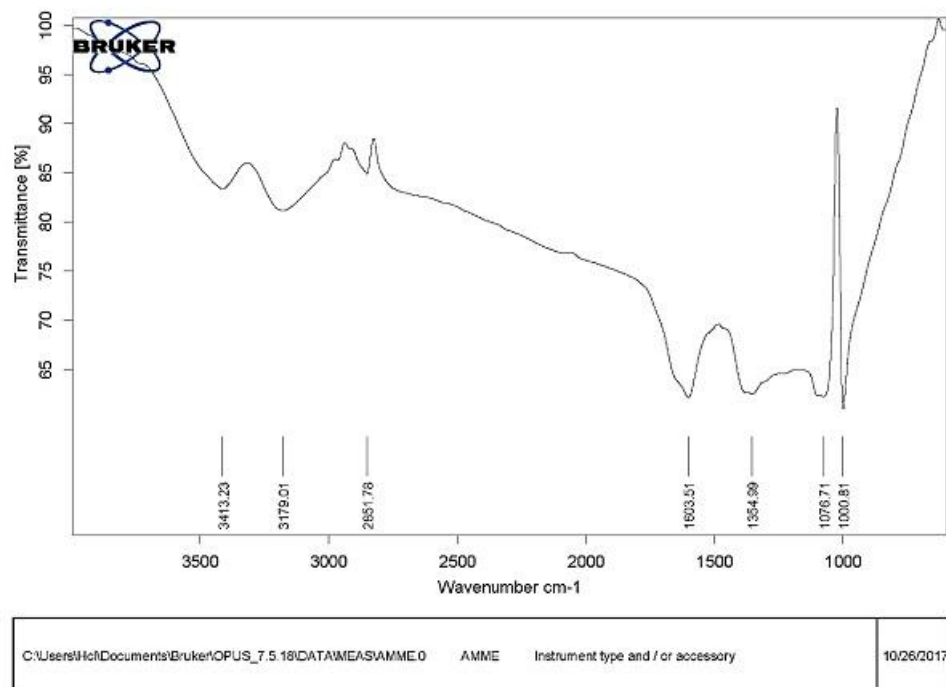


Figure 4: FTIR spectrum of *Argemone Mexicana* Linn methanol extract (AMME)

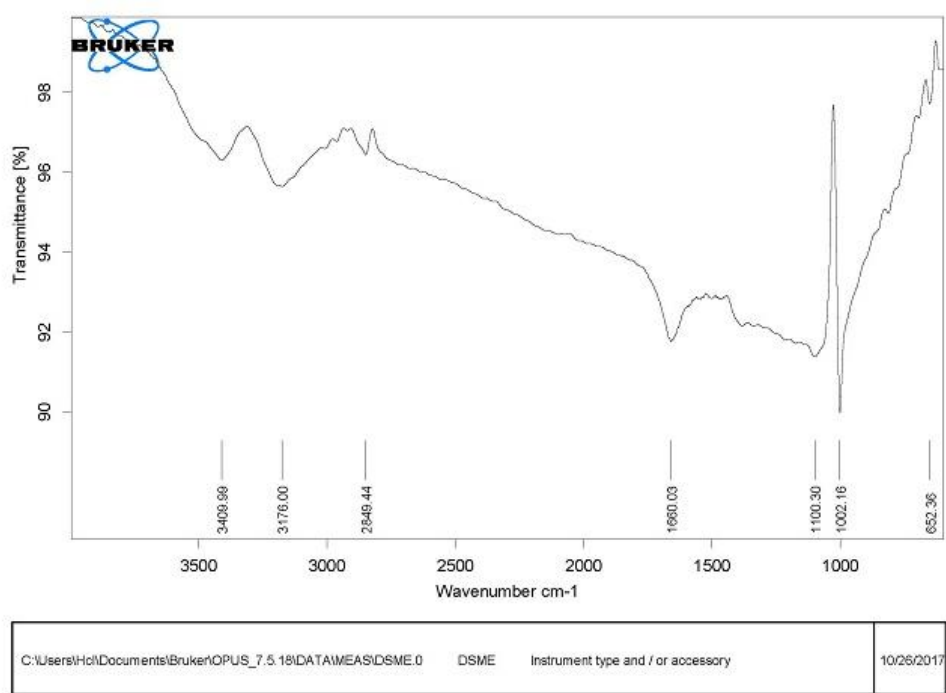


Figure 5: FTIR spectrum of *Datura stramonium* Linn methanol extract (DSME)

Galic acid and Rutin content

The percentage of Gallic acid contents for PZME, AMME and DSME was found to be $7.58 \pm 1.53\%$, $7.39 \pm 1.98\%$, and $9.18 \pm 1.41\%$ w/w respectively. The Rutin content for PZME, AMME and DSME was found to be $3.55 \pm 1.52\%$, $10.30 \pm 1.84\%$ and $13.26 \pm 1.65\%$ w/w respectively.

Thin layer chromatography of methanolic extracts

The R_f values obtained from PZME, DSME and AMME revealed the presence of alkaloids, tannins and phenols/

flavonoids. The R_f values obtained were matched with the standards and an inference was summarized in Table 2. The DSME revealed presence of tannins (4 spots) and flavonoids/phenolic compound (5 spots). 7-hydroxy flavone, Ferulic acid, 6-Hydroxy flavones and 6-Hydroxy flavones derivatives were inferred as phenolic and flavonoid compound. Similarly, cinnamic acid derivatives, Ferulic acid and paragalol were inferred as tannins based on their R_f values.

Table 2: R_f values of samples by TLC

Alkaloids (Cyclohexane: Chloroform: Diethyl amine 5:4:1)			
Sample extract	No. of spots	R_f value	Inference
AMME	1	0.511	Protropin derivatives
DSME	2	0.13	Scopolamine
		0.48	Scopoline derivatives
PZME	2	0.37	Plumbogin
		0.23	Napthaquinone derivatives
Tannins (Chloroform: Ethyl acetate: Acetic acid 50:50:10)			
AMME	3	0.48	Ferulic acid
		0.57	Cinnamic acid
		0.71	Cinnamic acid derivatives
DSME	4	0.76	Cinnamic acid derivatives
		0.53	Ferulic acid
		0.25	Paragallol
		0.68	Cinnamic acid derivatives
PZME	1	0.60	Cinnamic acid derivatives
Phenol/Flavonoid (Toluene: Ethyl acetate: Formic acid 36:12:5)			
AMME	3	0.56	Ferulic acid
		0.77	6-Hydroxy flavones
		0.88	Flavone
DSME	5	0.46	7-hydroxy flavone
		0.56	Ferulic acid
		0.67	6-Hydroxy flavones
		0.70	6-Hydroxy flavones derivatives
		0.88	Flavones
PZME	2	0.67	6-Hydroxy flavones
		0.70	6-Hydroxy flavones derivatives

Synergistic free radical scavenging activity of PHME

The results of the free radical scavenging activity of PZME, DSME, AMME and PHME tested by the DPPH method are depicted in Table 3. All test samples were found to exhibit increase in percentage inhibition with increase in concentration at λ_{max} 517 nm. On the other hand, the comparative antioxidant activity among the

test samples clearly revealed the combined synergistic effect of PHME, at the same concentration level selected for individual extract (PZME, DSME and AMME) with maximum % inhibition of 82.79% at 8 mg/ml as compared to standard ascorbic acid (92.92 %) as a standard antioxidant. The order of the antioxidant activity of test samples was found to be in order of PHME>PZME>AMME>DSME.

Table 3: Synergistic antioxidant effect of polyherbal methanol extracts (PHME)

Sample	% inhibition at different concentration			
	2mg/ml	4mg/ml	6mg/ml	8mg/ml
Ascorbic acid	63.84±1.263 b****	67.59±0.304 b***	87.26±1.909 b****	92.92±0.790 b****
PZME	20.52±0.397 a**** b****	56.77±0.447 a**** b*	57.82±0.599 a**** b****	74.13±0.520 a**** b****
DSME	17.09±1.064 a**** b****	44.44±0.150 a**** b****	47.77±1.611 a**** b****	75.35±1.267 a**** b****
AMME	13.77±0.728 a**** b****	45.88±0.193 a**** b****	61.93±0.220 a**** b****	78.64±0.939 a**** b*
PHME	40.43±0.872 a****	61.10±0.728 a***	71.78±1.599 a****	82.79±1.718 a****

All values are represented as mean \pm SEM, n = 3 for each group, Data were analyzed by two-way ANOVA, for each bacterial strain, followed by Dunnett's multiple comparisons test Multiple Comparisons Test, ****p < 0.0001 ***p < 0.001, **p < 0.01, *p < 0.1. (a) Denotes significant difference as compared to ascorbic acid (standard drug) and (b) denotes significant difference as compared to test drug PHME.

DISCUSSION

Phytochemical screening of the PHME showed the presence of triterpenoids, tannins, glycosides, flavonoids, polyphenols, carbohydrates, alkaloids etc. All these classes of compounds have good antioxidant potential and their effects on human nutrition and health are significant²⁰⁻²².

The FTIR spectrum of PZME, AMME and DSME showed peaks at 3408.82 cm⁻¹, 3143.23 cm⁻¹ and 3409.99 cm⁻¹ respectively corresponding with the hydroxyl groups of polyphenols while peaks at 1653.61cm⁻¹, 1603.51cm⁻¹ and 1660.03cm⁻¹ corresponds to carbonyl (C=O) groups. Sharp peaks at 1004.05cm⁻¹, 1000.81 cm⁻¹ and 1002.16 are assigned to alcohols (C-O) while peaks at 2851.78cm⁻¹, 2849.44 cm⁻¹ and 2847.88 cm⁻¹ showed the presence of carboxylic acid group. The majority of the peaks were broad and overlapped indicating the presence of a mixture of chemical constituents. The functional group may mask the other groups at a similar wave number. Peaks observed at 1653.61cm⁻¹, 1603.51cm⁻¹ and 1660.03cm⁻¹ corresponds to prominent flavonoids like quercetin and rutin which could be due to aromatic ketonic carbonyl stretching²³.

Many plant extracts have been shown to possess potent free radical scavenging activity that due to the combinatorial effect of polyphenolics and flavonoids. The flavonoids and polyphenols have the ability to induce human protective enzyme systems and are known to be a potent source of antioxidants. They exhibit protective effects against many infectious and degenerative diseases such as bacterial and viral diseases, cardiovascular diseases, cancers and other age-related diseases²⁴. Phytochemical characterization of DSME, AMME and PZME revealed the presence of Rutin (flavonoid) and Gallic acid (phenolic acid). The vicinyl dihydroxyl groups in Rutin and its metabolites were shown to affect the ability of phenols to inhibit iron and copper-catalyzed production of initiating radical species. Thus, it was more likely to scavenge radical by inhibiting glucose autoxidation²⁵. Among various polyphenols, Gallic acid (GA) (3,4,5-

trihydroxybenzoic acid) has been shown to possess strong antioxidant activities in many studies and was also found to exhibit both antioxidants as well as pro-oxidant characteristics²⁶.

The thin layer chromatography of the methanolic extract (DSME, PZME and AMME) selected for polyherbal combination revealed presence of alkaloid, tannins, polyphenol and flavonoid compounds²⁷. PHME was found to possess synergistic antioxidant activities as compared to individual constituents (DSME, PZME and AMME) and could be potentially used to potentiate tissue repair and rejuvenation process²⁸⁻²⁹. In living systems, free radicals are constantly generated and can cause extensive damage to tissues and biological molecules leading to various diseases. Many synthetic drugs can protect against oxidative damage, but, because of their adverse side effects an alternative solution is to consume natural antioxidants through food supplements and traditional medicine³⁰. Since ancient times, herbal or plant-based medicines have served as a platform for the prevention and cure of diseases and many constituents of these natural sources are yet to be explored. This has enlightened scientists to establish newer compounds alone or in combination of the herbal source having antioxidant activity to treat/mitigate oxidative stress induced pathogenesis for a number of diseases.

CONCLUSION

The polyherbal with its high antioxidant efficacy indicates that polyherbal supplement would become a significant source of natural antioxidants, helpful in preventing and mitigating harmful damage caused by oxidative stress.

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