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Secondary Group Analysis from Methanolic Extracts of Few Arthritic Plants and its *in vitro* Anti-Oxidant, Anti-Inflammatory Properties

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ABSTRACT

Background and Objective: Medicinal plants like *Cardiospermum halicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata, Vitex negundo* and their products are being used to treat various diseases worldwide, especially in Karnataka. This study aimed to investigate the few phytochemical constituents, *in vitro* antioxidant and anti-inflammatory activities of methanolic extracts of above mentioned plants. **Materials and Methods:** Extracts were screened to estimate presence of secondary metabolites like alkaloids, flavonoids and phenols. To check the antioxidant and anti-inflammatory potential total antioxidant capacity by phosphomolybdenum method was performed, H_2O_2 scavenging and Inhibition of protein denaturation and assay of cyclooxygenase, respectively. In the case of anti-inflammatory one nonsteroidal anti-inflammatory drug was used as reference and percentage inhibition of protein denaturation was calculated. Three different concentrations of extracts were prepared and analyzed for antioxidant studies taking ascorbic acid as standard. The IC_{50} value and percent inhibition from the formulation mixed from all the extracts in certain ratios were also evaluated. **Results:** The tested extracts showed excellent activities, whereas the formulated sample exhibited to be more potent than individual extract. Formulation is also found to contain large amounts of secondary metabolites such as alkaloids, flavonoids and phenols which increases the overall potency of formulation. The preparations of individual plants and their parts are employed traditionally for inflammatory and joint pain-related disorders. *In vitro* anti-inflammatory activity evaluated gives scientific validation and findings suggest that formulation can be a potent pain-relieving agent and also alleviate its use in oxidative stress-related diseases. **Conclusion:** Therefore, this would be a very promising source of treatment for the elimination of inflammation and arthritis.

KEYWORDS

Medicinal plants, alkaloids, *Cardiospermum halicacabum,* arthritis, flavonoids, phenols, *Vtex negundo,* oxidative stress

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INTRODUCTION

Antioxidants that are present in plants help in delaying cellular damage by their free radical scavenging property, by decreasing oxidative stress-induced carcinogenesis through inhibiting cell proliferation secondary to the protein phosphorylation[1,2]. In cases like excessive metabolism and oxidative stress, the

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generated free radicals inside our cells cause extensive damage to other nearby cells, DNA, mitochondria, etc. causing age-related degenerative diseases, cancer, diabetes, etc. Most of the antioxidants that arrive from plants include vitamins like A, C and E, carotenoids like beta-carotene, minerals, phenolic compounds and many other natural chemicals that have antioxidant properties[3].

Rheumatoid arthritis is considered a chronic, disabling and progressive autoimmune disorder in which chronic proliferative synovitis and inflammation in synovial are observed with significant destruction in bone and cartilage leading to damage to joints and reduced functionality[4-6]. This type of pathology is known to evolve very quickly in every individual affecting several parts of the body which causes extreme pain in individuals suffering from degenerative bone disorders and dysfunctioning immune system[7]. This pathology also occurs due to the attack on immune system, instability in synovial membrane along with swelling, stiffness, pain and loss of joint functioning[7]. During this developing phase of rheumatoid arthritis, there lies several inflammatory mediators that play major role in destruction of bone and inflammation of the synovial membranes from tumor necrosis factor (TNF-α), interleukin-6, interleukin-1β, nitric oxide (NO), reactive oxygen species (ROS), prostaglandins, platelet-activating factor, leukotrienes, many enzymes mainly (lipoxygenases, cyclooxygenases (COX-1 and COX-2) and phospholipases)[8,9].

In continuous surveys using bioactive extracts from plants used in traditional medicine, it is found that plants such as *Cardiospermum helicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata* and *Vitex negundo* are employed so far for treating severe arthritic pains since time immemorial. In this regard to support and improve the traditional use of these plants, we have already investigated anti-arthiritc properties and its thorough bioactive compounds analysis through GCMS. Here studies are undertaken to carry out the *in vitro* anti-inflammatory and antioxidant activities and their estimation of alkaloids, flavonoids and phenols. Also, attempt is made to improve the overall effect of these plant extracts by formulating the mixture in different ratios and analysing its anti-inflammatory potential.

MATERIALS AND METHODS

Plant collection and preparation of extract: The leaves of *Cardiospermum halicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata* and *Vitex negundo* were collected in and around Dakshina Kannada District of Karnataka in June 2023. The collected samples were washed thoroughly with distilled water and shade-dried for 5 day at room temperature (37°C). The plants were authenticated and identified using DNA barcoding technique. These dried leaves were finely ground using an electric grinder (BOSCH Professional GWS 600 Angle Grinder - 670W) and pulverized plant material (10 g) was extracted twice by soaking with 100 mL of methanol for 48 hrs at room temperature. Further, the separated extracts were filtered through Whatman No. 1 filter paper and the methanol filtrate was condensed to dryness using a rotary evaporator (Buchi) at 40°C. This dried extract was taken for further studies.

Screening for phytochemicals: Phytochemical analysis of methanol extract of all the plants individually and from formulation was carried out for the detection of active secondary metabolites like alkaloids, flavonoids and phenolic compounds by adopting standard methods.

Estimation of alkaloids: Alkaloids were determined in plant extracts and formulated samples using Harborne gravimetric method[10]. For quantitative test 2 gm of sample was taken in 10 mL of ethanol. Shaken vigorously for a few minutes, filtered the mixture using Whatman No. 40 filter paper. The 2 mL of filtrate was taken into test tube, added 3 drops of picric acid and mixed thoroughly. The formation of light green coloration indicated the presence of alkaloids. For quantitative determination of alkaloids 5 gm of sample was taken and 200 mL of 20% acetic acid was added. Mixture was kept at room temperature for 4 hrs and filterate was concentrated over a steam bath. After cooling ammonium hydroxide was added

dropwise to precipitate the alkaloid in the extract solutions. This precipitate of alkaloids was filtered, washed with 9% ammonium solution and dried in oven (Panasonic) at 60°C for 30 min. This mixture was cooled in a desiccator (Borosil) and weighed for the estimation of alkaloids.

Estimation of flavonoids: The qualitative analysis of flavonoids was performed in test tubes by adding 1 mL of respective extracts and a few drops of 10% NaOH. The change in colour to yellow signified the presence of flavonoids in the respective sample. Whereas for quantitative analysis of flavonoids 1 mL of respective extracts and 10 mL of 80% ethanol were added in petri dishes. The weight of petri dishes was taken before (SW) and after overnight drying (DW) and flavonoid content was estimated.

Estimation of phenols: The qualitative analysis of phenols was conducted in test tubes by taking 1 mL of respective samples and adding a few drops of Gram's iodine to it. The formation of reddish-brown colour indicated the presence of phenols. For the quantitative analysis of phenols 0.5 mL of sample was taken in test tubes, 3.75 mL distilled water, 0.25 mL Folin-Ciocalteu (FC) reagent and 0.5 mL of 35% Na_2CO_3 was added to it. After incubating the samples at room temperature for 90 min, absorbance was measured at 765 nm.

***In vitro* anti-oxidant activity**

H_2O_2 scavenging test: To 1mL of sample 5 mL of 50 mM pH 7.4 phosphate buffer and 4 mL of 2 mM H_2O_2 was added and the tubes were incubated for 10 min. The absorbance for each extract was measured at 510 nm, taking phosphate buffer without H_2O_2 as blank. Gallic acid was used as a standard drug. The amount of hydrogen peroxide scavenged was obtained from the following equation:[11]

$$H_2O_2 \text{ scavanged} = \frac{OD_{test}}{OD_{control}} \times 100$$

Phosphomolybdenum method (total antioxidant capacity-TAC): To 500 µL of each plant extract 3 mL of phosphomolybdenum reagent was added. The tubes were I ncubated in boiling water bath at 95 for 90 min. The absorbance of each sample was observed at 695 nm with blank (1 mL of solvent in place of the sample was taken). The 0.588 mL of H_2SO_4, 0.049 g ammonium molybdate and 0.036 g sodium phosphate were added together to prepare phosphomolybdenum reagent.

***In vitro* anti-inflammatory activity**

Inhibition of protein denaturation: To evaluate the *in vitro* anti-inflammatory effects of the samples modified protocol is followed[12,13]. The 1 mL of extracts at different concentrations (125, 250 and 500 µg/mL) was homogenised with 1 mL of aqueous solution of bovine serum albumin (5%) and incubated at 27°C for 15 min. Diclofenac sodium was used as a positive control, mixture of distilled water and BSA constituted the negative control. Denaturation of the proteins was caused by placing the mixture in a water bath for 10 min at 70°C. This mixture was cooled inside the ambient temperature and the activity was measured at 660 nm in triplicates[14].

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

RESULTS AND DISCUSSION

The plant samples extracted from ethanol were investigated for the presence of phytochemicals such as alkaloids, flavonoids and phenols. They have shown increased concentrations in case of formulation, as shown in Table 1 and 2. The preliminary qualitative analysis indicated the presence of alkaloids, flavonoids and phenols in all the extracts as well as formulation. The quantitative phytochemical analysis of all plant

Table 1: Qualitative screening of phyto-chemicals in ethanolic extracts of plants and their formulation

Sample	Alkaloids	Flavonoids	Phenols
Cardiospermum halicacabum	++	+++	+
Kirganelia reticulata	+	++	++
Pongamia pinnata	++	+++	+
Scoparia dulcis	++	+++	++
Urena lobata	+	+	+
Vitex negundo	++	++	++
Formulation	+++	+++	+++

+: Partially present, ++: Present and +++: Present in good quantity

Table 2: Quantitative screening of phyto-chemicals in ethanolic extracts of plants and their formulation

Sample	Alkaloids mg A/mL	Flavonoids mg EA/mL	Phenols mg GAE/mL
Cardiospermum halicacabum	156.82	498.45	167.89
Kirganelia reticulata	98.45	325.14	245.67
Pongamia pinnata	136.78	468.35	152.78
Scoparia dulcis	142.27	478.13	267.18
Urena lobata	87.25	256.12	141.92
Vitex negundo	156.26	312.87	287.14
Formulation	237.8	550.83	356.91

Table 3: H_2O_2 scavanging and total antioxidant capacity of methanolic extracts of plants and their formulation

Treatment	OD values at 510 nm	H_2O_2 scavanging (%)	Total antioxidant capacity (695nm)
Cardiospermum halicacabum (125µg/mL)	0.13	31.70±1.13	0.312
Cardiospermum halicacabum (125µg/mL)	0.25	60.97±1.06	0.267
Cardiospermum halicacabum (125µg/mL)	0.31	75.60±0.80	0.341
Kirganelia reticulata (125µg/mL)	0.16	39.02±0.97	0.169
Kirganelia reticulata (205µg/mL)	0.27	65.85±0.43	0.285
Kirganelia reticulata (500µg/mL)	0.32	78.04±0.81	0.298
Pongamia pinnata (125µg/mL)	0.21	51.21±1.02	0.209
Pongamia pinnata (250µg/mL)	0.33	80.48±0.64	0.346
Pongamia pinnata (500µg/mL)	0.37	90.24±1.04	0.335
Scoparia dulcis (125µg/mL)	0.18	43.90±0.88	0.198
Scoparia dulcis (250µg/mL)	0.22	53.65±0.84	0.216
Scoparia dulcis (500µg/mL)	0.26	63.41±0.27	0.259
Urena lobata (125µg/mL)	0.16	39.02±1.15	0.169
Urena lobata (250µg/mL)	0.23	56.09±0.71	0.227
Urena lobata (500µg/mL)	0.29	70.73±0.64	0.284
Vitex negundo (125µg/mL)	0.18	43.90±0.72	0.197
Vitex negundo (250µg/mL)	0.31	75.60±0.74	0.298
Vitex negundo (500µg/mL)	0.35	85.36±1.87	0.348
Formulation(125 µg/mL)	0.351	85.60±1.79	0.359
Formulation(250 µg/mL)	0.372	90.73±1.72	0.372
Formulation(500 µg/mL)	0.386	94.14±1.36	0.391
Negative control	0.41	-	0.205

Each value represents Means±SD (n = 3)

extracts revealed a promisable percentage of alkaloids, flavonoids and phenols. Greater amounts of alkaloids were present in *Cardiospermum halicacabum, Pongamia pinnata, Scoparia dulcis* and *Vitex negundo*. Higher amounts of flavonoids were detected in *Cardiospermum helicacabum, Pongamia pinnata* and *Scoparia dulcis,* followed by *Kirganelia reticulata* and *Vitex negundo.* In the same way phenol content was found to be more in *Kirganelia reticulata, Scoparia dulcis* and *Vitex negundo*. Whereas all three metabolites were found to be highest in the formulation as it is attributed to combined effect of all plant extracts. In formulation alkaloid was found to be 237.8 mg AE/mL, where Atropine was used as standard; Flavonoid was found to be 550.83 mgEAE/mL, where Ellagic acid was used as standard; Phenol was found to be 356.91 mgGAE/mL, where Gallic acid was used as standard.

Table 4: percentage inhibition of protein denaturation in methanolic extracts of plants and their formulation

Treatment	OD values at 660 nm	Protein denaturation inhibition (%)
Cardiospermum halicacabum (125µg/mL)	0.030	83.33±0.90
Cardiospermum halicacabum (250µg/mL)	0.027	85.21±1.07
Cardiospermum halicacabum (500µg/mL)	0.013	92.77±1.12
Kirganelia reticulata (125µg/mL)	0.031	82.77±0.91
Kirganelia reticulata (205µg/mL)	0.028	84.44±0.41
Kirganelia reticulata (500µg/mL)	0.014	92.2±0.87
Pongamia pinnata (125µg/mL)	0.038	78.88±1.01
Pongamia pinnata (250µg/mL)	0.033	81.66±0.63
Pongamia pinnata (500µg/mL)	0.021	88.33±1.12
Scoparia dulcis (125µg/mL)	0.041	77.22±0.29
Scoparia dulcis (250µg/mL)	0.036	80±0.98
Scoparia dulcis (500µg/mL)	0.024	86.66±0.81
Urena lobata (125µg/mL)	0.033	81.66±0.73
Urena lobata (250µg/mL)	0.029	83.88±0.69
Urena lobata (500µg/mL)	0.016	91.11±1.19
Vitex negundo (125µg/mL)	0.035	80.55±1.92
Vitex negundo(250µg/mL)	0.031	82.77±0.79
Vitex negundo (500µg/mL)	0.018	90±0.92
Diclofenac sodium (50 µg/mL)	0.002	98.88±0.21
Formulation (125 µg/mL)	0.0243	86.66±0.8
Formulation (250 µg/mL)	0.0187	90±0.82
Formulation (500 µg/mL)	0.0131	92.77±1.02
Negative control	0.18	-

Each value represents Means±SD (n = 3)

For estimating the antioxidant activity, the absorbance of plant extracts at 510 nm was analysed along with formulation, respectively. Here also, in total formulated sample showed higher H_2O_2 scavenging percentage when compared to individual plant extracts which were estimated in 3 different concentrations (125, 250 and 500 µg/mL). The antioxidant activity was found to be in dose-dependent manner in all the plant extracts. The same pattern of effect was found in phosphomolybdenum method which estimated total antioxidant capacity. The absorbance of H_2O_2 scavenging and its percentage and total antioxidant capacity are as shown in Table 3. While conducting *in vitro* anti-inflammatory activity percentage inhibition of protein denaturation was estimated, where all the plant extracts showed moderate and dose-dependent effects. Whereas formulated extract effectively inhibited protein denaturation (albumin) caused by heat as shown in Table 4, of up to 92.77%.The positive control diclofenac sodium produced 98.88% inhibition. In addition, the inhibition rate of formulated extract increased with the increase in concentration which is promising and found to be significant with standard.

DISCUSSION

The present study indicated the presence of secondary metabolites, antioxidant and anti inflammatory property of individual plant extracts as well as their formulations in high quantity. The high amount of alkaloids present in formulation is attributed to the fact that the high solubility of the alkaloids in the combined extract rather than in individual extracts[15,16]. It is said that the high solubility of phenols in polar solvents reflects high concentrations in extracts and the same trend is followed in the present investigation where combined extracts have increased the solubility of phenolic compounds[17]. In various studies it is mentioned that the concentration of flavonoid in plant extracts depends on the polarity of solvents used in the extract preparation and so is our results depicting[18]. Phenolic and flavonoid compounds present in plants are said to correlate with antioxidant activities. Based on the estimation of total alkaloids, flavonoids and phenols, further investigation of antioxidant studies has proved to be perfectly correct. The antioxidant studies revealed a higher percentage of scavenging activity i.e. due to their redox properties, playing an important role in absorbing, neutralizing free radicals and decomposing peroxides. In this regard, two different methods followed revealed maximum antioxidant activity[19]. Free radicals thereby refer to the reactive atoms or groups of atoms having one or more electrons which get produced in body

either through natural biological processes or introduced from outside sources by damaging cells, DNA and proteins by altering their chemical structures. There are several free radical scavenging methods that are employed for the detection of antioxidant activity[20]. Here the followed method employs free radicalscavenging ability of the sample measuring antioxidant activity[21]. Naturally-occurring iron complexes present inside the cell react with H_2O_2 *in vivo* and highly reactive hydroxyl radicals are generated, causing the origins of toxic effects[22].

Denaturation of proteins is the process in which they lose their tertiary and secondary structures which is well-documented in case of inflammation[23]. This mechanism alters a series of actual events and causes events in which the metabolism of arachidonic acid plays major role. Here denaturation of proteins shows that these plant extracts and formulations are capable of significantly inhibiting the production of prostaglandins and leukotrienes, giving anti-inflammatory properties to extracts. Several previous studies have done analysis and anti arthritic properties of these extracts are proven. These results support the traditional uses of these plants in some painful, inflammatory and arthritic conditions. So here it is not able to claim that because of the presence of biologically active principles, i.e. alkaloids, flavonoids and phenolic compounds phytochemical investigation, it is assumed that may be due to any one of the above constituents or in combination together from all plants is responsible for showing anti inflammatory effects. The formulation done with these plant extracts also suggested that it may have a reasonable safety margin with regard to acute toxicity, anyhow further justifying its wide application in various communities and investigation of side effects is a must[23]. Therefore, this study brings out the potentiality of individual plant and collective improved effect when formulated together in particular ratio with anti-inflammatory effects, supporting strongly its antiarthritic claims.

CONCLUSION

The present study attributes this to the fact that plants have potent phytochemicals having therapeutic activities due to the presence of secondary metabolites and have significant antioxidant and anti-inflammatory activity due to estimated total alkaloid, flavonoid and phenol content. The formulation here played a vital role in enhanced activities in which the combined effect of phytochemicals has been attributed to significant antioxidant and anti-inflammatory activity. The antioxidants derived from plant origin possessing free radical scavenging properties exhibit great importance as therapeutic agents in treating several diseases that are caused due to oxidative stress. Therefore, the results obtained in the present study indicate that *Cardiospermum halicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata* and *Vitex negundo* extracts have good potential to act as a source of useful drugs due to the presence of various phytochemical components in it. Their effect has been drastically enhanced when combined in the form of formulation. Further studies aiming towards its toxicity and dosage level for oral consumption are in progress.

SIGNIFICANCE STATEMENT

The present study deals with the investigation of the leaves of *Cardiospermum halicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata, Vitex negundo* for their anti-arthritic potential, as they are widely used in traditional medicinal systems of for the treatment of arthritis. The present study concludes the use of formulation from these plants as an effective agent against arthritis and further investigational studies are required to identify the exact phytoconstituents responsible from each plant for the activity and also to elucidate the precise mechanism which exhibits the anti-arthritic activity of individual plants and their formulation.

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