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In vitro Anti - Arthritic Studies of Few Medicinal Plants having Traditional Claim for Treating Arthritis in Dakshina Kannada, Karnataka

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Abstract: ***Use of herbal medicine is becoming popular due to its less toxicity and side effects when compared to allopathic medicines. The plants such as Cardiospermum helicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata and Vitex negundo acts as one of the important sources maintaining its place in treating arthritis with minimum scientific validation. Considerable studies have been carried out on these ethno medicinal plants but very few of them have attracted the interest of scientists, to investigate them as cure for arthritis. Our present work aims at investigating the in - vitro anti - arthritic effects of Cardiospermum helicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata and Vitex negundo at various concentrations. The inhibition of protein denaturation, membrane stabilization and proteinase inhibitory action were taken as a measure of the in - vitro anti - arthritic activity. The same effects were investigated for the formulation prepared from these extracts. The maximum anti - arthritic effect was shown by Cardiospermum helicacabum and Kirganelia reticulata at dose dependent manner. This is followed by Urena lobata and Vitex negundo showing good effect, which is then followed by Pongamia pinnata and Scoparia dulcis. Interestingly the formulation had shown excellent effects when compared to standard acetyl salicylic acid. Therefore, our studies support the traditional usage of above plants for treating arthritis. This study not only confirms the ethnomedicinal use of above plants in the treatment of arthritis, but also indicates the strong anti - arthritic effects when culminated together. Additional clinical trials are however required further to show the effectiveness of formulation in different immuno - inflammatory conditions.***

Keywords: protein denaturation, membrane stabilization, proteinase inhibitory action, formulation

1. Introduction

Pharmacology is the scientific Knowledge of action and effects of drugs on living systems and the interaction of these drugs with living systems. Arthritis is inflammatory, chronic, systemic autoimmune disease which is characterized by pain, stiffness and swelling (Shivanand, 2010). In India approximately more than 20% of total population is suffering from arthritis. The joints that are commonly affected by arthritis are weight-bearing joints like feet, hips, knees, spine and other joints such as finger, thumb etc. Allopathic medications have been prescribed to lighten the symptoms of this disease resulting in associated side effects like heart attack, stomach ulcers, stroke, bleeding from digestive tract, kidney damages etc (Sunetra et al., 2010). Unfortunately, still there is no effective known medicinal treatment that cures rheumatoid arthritis which highlights possible usage of herbs and plants in various forms to relieve pain and inflammation in joints. There are so many medicinal plants that have shown anti rheumatoid arthritis properties and their plant products being have significant advantages for treating Rheumatoid arthritis. Many antiarthritic plants have shown to contain a variety of chemical constituents like alkaloids, phenols, essential oils, coumarins, catechins, monoterpenes, quinones, flavanoids, carotinoids, anthocyanins, xanthenes etc (Shah et al., 2006).

Few most common forms of arthritis comes under the category of inflammatory immune arthritis (IIA) (Menegatti et al., 2019). Here cytokines, oxygen derivatives, proteinases and interleukins (IL) acts as inflammatory mediators found in blood plasma and synovial fluid during arthritis, which are linked to inflammation and cartilage destruction. These mediators are thus synthesized by immune cells and hence, released into inflamed joint (Pietrosimone et al., 2015). In the pathogenesis of rheumatoid arthritis, a significant role of NF - κB signaling has been elucidated (Brücher et al., 2019). Whereas, in the nuclear DNA responsive element to NF - κB is transcribed producing cytokines such as tumor necrosis factor - α (TNF - α), IL - 6, and IL - 1β (McDaniel et al., 2016). Therefore, primary inflammatory reaction is due to the synthesis and release of histamine, leukotrienes and prostaglandins (PG). These PG's (PGG2 and PGH2) gets synthesized from a fatty acid - derived substance arachidonic acid by COX - 2 enzyme with response to immunological and chemical stimuli. The anti - inflammatory effect of many plants and phytomedicines is due to the inhibition of COX - 2 and IL (Brown N, Roberts, 2001). The production of auto antigens in few specific arthritic diseases are due to *in vivo* denaturation of proteins (Brown J. H., Mackey, 1968). The mechanism of denaturation followed here probably involves alteration in electrostatic, hydrophobic, hydrogen and disulphide bonding (Grant et al., 1970). Hence, by controlling the production of auto antigen and inhibiting denaturation of protein and membrane lysis in arthritic diseases leads to anti - arthritic activity. Therefore, inhibition of protein denaturation and membrane lysis can be taken as a measure of the in vitro anti - arthritic activity.

In this case, our study is focused on investigating the medicinal plants extracts against *in vitro* anti arthritis activity. The plants such as *Cardiospermum helicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis,*

Urena lobata and *Vitex negundo* that are available in Dakshina Kannada, Karnataka and have traditional claim for treating arthritis are taken. The common problem associated with using plants as herbal medicine is adulteration and use of spurious materials as substitute. Such adulterated herbal drugs are dangerous to human health and cause various health issues. Hence, individual plant extracts as well as formulation prepared from above plant extracts are checked for *in vitro* anti arthritic potential *at* various concentrations by using *in - vitro* pharmacological models.

2. Materials and methods

Collection of Plant Material

The fresh leaf samples of selected medicinal plants *Cardiospermum helicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata* and *Vitex negundo* were collected in and around Dakshina Kannada, Karnataka by discussing with people who use it in traditional medicine for treating arthritis. The leaves were surface sterilized to remove dirt and other impurities.

Preparation of ethanolic extracts

Ethanolic extracts were prepared using cold extraction method aiming towards extracting maximum components from the plant.10 gm of leaf samples were cut into small pieces and soaked in 50ml of ethanol keeping at room temperature. After 7 - 8 days once the materials became colourless, the extract was filtered and filtrate was concentrated using water bath to get the crude extract which was used for In vitro antiarthritic activities. Individual plant extracts were checked separately for the effect and then all the extracts were mixed in equal ratio and developed a formulation to check the same activites.

***In vitro* antiarthritic activities**

Inhibition of protein denaturation:

The assay was conducted as per modified method of Sharan et al., 2011. The reaction mixture consisted of 0.45 ml bovine serum albumin (5%W/V aqueous solution) and 500 µl of plant extracts (125, 250 and 500 µg/ml) in DMSO. The samples were incubated at 37^{o}C for 20 min and then heated at 57^{o}C for 3 min. After cooling the samples, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660 nm, for control tests 500 µl of distilled water was used instead of compound. The percentage inhibition of protein denaturation (Snedecor and Cochran 1967) was calculated as follows:

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

The control represents 100% protein denaturation. The results were compared with acetyl salicylic acid (50 µg/ml) treated samples.

Effect on membrane stabilization:

The assay was conducted as per modified method of Sharan et al., 2011. The principle involved here is stabilization of human red blood cell (HRBC) membrane by hypo tonicity induced membrane lysis. The reaction mixtures consisted of 2 ml hypotonic saline (0.25% NaCl), 1 ml 0.15 M phosphate buffer (pH 7.4) and 1 ml of plant extracts (125, 250 and 500 µg/ml).0.5 ml of 10% rat RBC in normal saline was added. For control tests, 1 ml of isotonic saline was used instead of test solution and the mixtures were incubated at 56^{oC} for 30 mins. The tubes were cooled under running tap water for 20 mins, centrifuged and the absorbance of the supernatants read at 560 nm. Percent membrane stabilizing activity (Mizushima 1966) was calculated as follows:

$$\text{Percent membrane stabilization} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}}\right) \times 100$$

The control represents 100% lysis. The results were compared with acetyl salicylic acid (50 µg/ml) treated samples.

Proteinase inhibitory action:

The assay was conducted as per the method followed by Vallabh et al. (2009). The reaction mixtures contained 0.06 mg trypsin, 1.0 ml 25 mM tris - HCl buffer (pH 7.4) and 1 ml of plant extracts (125, 250 and 500 µg/ml) in DMSO. The mixtures were incubated at 37^{o}C for 5 mins and then 1.0 ml of 0.8% (w/v) casein was added. The mixtures were incubated for additional 20 mins and 2.0 ml of 70% (v/v) perchloric acid was added to terminate the reaction. The cloudy suspension was centrifuged and absorbance of the supernatant was read at 280 nm against buffer as blank. The percentage of proteinase inhibition (Brown and Mackey 1968) was calculated as follows:

$$\text{Percent proteinase inhibition} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}}\right) \times 100$$

The control represents 100% inhibition. The results were compared with acetyl salicylic acid (50 µg/ml) treated samples.

3. Results and Discussion

Since we have selected plants that are used for treating arthritis in traditional medicine, all the plants have showed excellent activities in all the in vitro models. Among the plants taken for study, the ethanolic extract of *Cardiospermum helicacabum* has showed significant activity at all three concentrations and its effect was compared with the standard drug acetyl salicylic acid. The maximum percentage inhibition of protein denaturation and membrane stabilisation of *Cardiospermum helicacabum* was observed to be more than 95% at 500µg/ml, which is followed by *Kirganelia reticulata* as shown in **table 1**, **table 2** and **table 3**. Followed by these plants *Urena lobata* and Vitex *negundo* have shown moderate results in all the experiments at 3 different concentrations 125, 250 and 500 µg/ml. The last but still efficient activity had been shown by extracts of *Pongamia pinnata* and *Scoparia dulcis.* All the plant extracts have shown effect in dose dependent manner and consistent results when compared to standard drug. Interestingly, the formulation prepared from all 6 plants has shown increased and excellent effect when compared to

acetyl salicylic acid, and again the effects are in dose dependent manner as shown in **table 4**. The production of auto antigen in arthritic diseases is attributed to denaturation of protein and lysing of membrane. From the results obtained in our present study, it can be stated that ethanolic extracts, especially formulation is capable of controlling the production of auto antigen inhibiting denaturation of protein and membrane lysis in rheumatoid arthritic diseases. Here, inhibition of protein denaturation and membrane stabilisation were studied to understand the mechanism of anti - arthritic effects of above listed plants. Therefore, the significant anti - arthritic activity demonstrated by plant extracts and formulation can be due to the presence of active principles like flavonoids, alkaloids, tritrepenoids, bacosides and other related polyphenols showing responsibility of this activity (Sharan et al., 2011). Hence, as the formulation has shown promising results, it can be considered as potent anti - arthritic agent.

Studies done by Eswar et al., (2008) suggest that the ethanolic extract of *Cardiospermum halicacabum* leaves exhibits significant anti - arthritic effects in Freund's complete adjuvant (FCA) induced arthritis in rats. Shruthi et al., (2012) have tested *Kirganelia reticulata plant extracts and isolated compounds on in vitro, in vivo, in silico models giving scientific validation and* supporting the traditional use of plant for rheumatism. Study was conducted by Rajagopal et al., (2019) to ascertain the *in vitro* anti - arthritic activity of the aqueous extract of leaves of *Urena lobata*. They have evaluated protein denaturation, where plant produced remarkable anti - arthritic activity. As *Vitex negundo* is traditionally used as antiarthritic, antiinflammatory, antileprotic, anti - asthmatic, anti - spasmodic, astringent and anthelmintic agent, Pavithra et al., (2015) aimed towards investigating its in vitro antiarthrtic activity of aqueous extract by denaturation of protein methods. Aqueous extract of *Vitex negundo* was screened for antiarthritic activity by denaturation of egg albumin and bovine albumin proteins which showed remarkable *in vitro* antiarthritic effect against the denaturation of protein. Different leaf extract such as aqueous, alcoholic, acetone and chloroform of *Pongamia pinnata* were investigated for *in - vivo* anti - inflammatory activity by carrageenan induced paw edema model by Rashmi Sagar et al., (2018). The extracts showed dose dependent anti - inflammatory activity with maximum reduction in oedema observed in alcoholic extract. Scoparia dulcis has been extensively used to relieve discomfort caused by various painful conditions. Hence, with the objective to evaluate the analgesic and anti - inflammatory effect the crude extract of S. dulcis in experimental model of osteoarthritis was performed by Marcus et al., (2019). Their results showed that 15 - days treatment with crude extract reduces edema, peripheral nociceptive activity, spontaneous pain and proinflammatory cytokines in the synovial fluid. The highest inhibition of cyclooxygenase 2 was observed in crude extracts thus presents *S. dulcis* to have therapeutic potential for treating osteoarthritis due to its anti - inflammatory and anti - nociceptive action.

Table 1: Showing percentage inhibition of protein denaturation in ethanolic extracts of *Cardiospermum helicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata* and *Vitex negundo* at 125, 250 and 500 µg/ml concentrations. Each value represents means ± SD (n=3).

Sl. No.	Treatment	OD values at 660 nm	Protein Denaturation inhibition (%)
1	*Cardiospermum helicacabum (*125µg/ml)	0.030	83.33±0.90
2	*Cardiospermum helicacabum (*250µg/ml)	0.027	85.21±1.07
3	*Cardiospermum helicacabum (*500µg/ml)	0.013	92.77±1.12
4	*Kirganelia reticulata* (125µg/ml)	0.031	82.77±0.91
5	*Kirganelia reticulata* (205µg/ml)	0.028	84.44±0.41
6	*Kirganelia reticulata* (500µg/ml)	0.014	92.2±0.87
7	*Pongamia pinnata* (125µg/ml)	0.038	78.88±1.01
8	*Pongamia pinnata* (250µg/ml)	0.033	81.66±0.63
9	*Pongamia pinnata* (500µg/ml)	0.021	88.33±1.12
10	*Scoparia dulcis* (125µg/ml)	0.041	77.22±0.29
11	*Scoparia dulcis* (250µg/ml)	0.036	80±0.98
12	*Scoparia dulcis* (500µg/ml)	0.024	86.66±0.81
13	*Urena lobata* (125µg/ml)	0.033	81.66±0.73
14	*Urena lobata* (250µg/ml)	0.029	83.88±0.69
15	*Urena lobata* (500µg/ml)	0.016	91.11±1.19
16	*Vitex negundo* (125µg/ml)	0.035	80.55±1.92
17	*Vitex negundo* (250µg/ml)	0.031	82.77±0.79
18	*Vitex negundo* (500µg/ml)	0.018	90±0.92
19	Acetyl Salicylic acid (50 µg/ml)	0.002	98.88±0.21
20	Negative control	0.18	-

Table 2: Showing percent membrane stabilizing activity in ethanolic extracts of *Cardiospermum helicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata* and *Vitex negundo* at 125, 250 and 500 µg/ml concentrations. Each value represents means ± SD (n=3).

Sl. No.	Treatment	OD values at 560 nm	Membrane Stabilitization (%)
1	*Cardiospermum helicacabum (*125µg/ml)	0.025	85.29±0.89
2	*Cardiospermum helicacabum (*250µg/ml)	0.022	87.05±0.99
3	*Cardiospermum helicacabum (*500µg/ml)	0.009	94.70±0.82
4	*Kirganelia reticulate* (125µg/ml)	0.027	84.11±0.79
5	*Kirganelia reticulate* (205µg/ml)	0.022	87.05±0.81
6	*Kirganelia reticulata* (500µg/ml)	0.009	94.70±0.59

7	*Pongamia pinnata* (125µg/ml)	0.033	80.58±1.09
8	*Pongamia pinnata* (250µg/ml)	0.027	84.11±0.81
9	*Pongamia pinnata* (500µg/ml)	0.016	90.58±0.78
10	*Scoparia dulcis* (125µg/ml)	0.037	78.23±0.82
11	*Scoparia dulcis* (250µg/ml)	0.031	81.76±0.92
12	*Scoparia dulcis* (500µg/ml)	0.020	88.23±0.74
13	*Urena lobata* (125µg/ml)	0.028	83.52±0.82
14	*Urena lobata* (250µg/ml)	0.023	86.47±0.93
15	*Urena lobata* (500µg/ml)	0.011	93.52±0.81
16	*Vitex negundo* (125µg/ml)	0.030	82.35±0.94
17	*Vitex negundo* (250µg/ml)	0.026	84.70±0.86
18	*Vitex negundo* (500µg/ml)	0.012	92.94±1.02
19	Acetyl Salicylic acid (50 µg/ml)	0.01	94.11±1.19
20	Control	0.17	-

Table 3: Showing percentage of proteinase inhibition in ethanolic extracts of *Cardiospermum helicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata* and *Vitex negundo* at 125, 250 and 500 µg/ml concentrations. Each value represents means ± SD (n=3).

Sl. No.	Treatment	OD values at 280 nm	Proteinase inhibition (%)
1	*Cardiospermum helicacabum* (125µg/ml)	0.023	88.5±0.71
2	*Cardiospermum helicacabum* (250µg/ml)	0.021	89.5±0.89
3	*Cardiospermum helicacabum* (500µg/ml)	0.007	96.5±0.73
4	*Kirganelia reticulate* (125µg/ml)	0.025	87.5±0.82
5	*Kirganelia reticulate* (205µg/ml)	0.020	90.0±0.79
6	*Kirganelia reticulate* (500µg/ml)	0.007	96.5±0.91
7	*Pongamia pinnata* (125µg/ml)	0.031	84.5±0.92
8	*Pongamia pinnata* (250µg/ml)	0.025	87.5±0.82
9	*Pongamia pinnata* (500µg/ml)	0.014	93.0±0.74
10	*Scoparia dulcis* (125µg/ml)	0.035	82.5±1.12
11	*Scoparia dulcis* (250µg/ml)	0.029	85.5±1.09
12	*Scoparia dulcis* (500µg/ml)	0.018	91.0±0.95
13	*Urena lobata* (125µg/ml)	0.026	87.0±0.98
14	*Urena lobata* (250µg/ml)	0.021	89.5±0.81
15	*Urena lobata* (500µg/ml)	0.009	95.5±0.84
16	*Vitex negundo* (125µg/ml)	0.028	86.0±0.93
17	*Vitex negundo* (250µg/ml)	0.024	88.0±0.84
18	*Vitex negundo* (500µg/ml)	0.011	94.5±0.79
19	Acetyl Salicylic acid (50 µg/ml)	0.005	97.5±1.03
20	Control	0.20	-

Table 4: Showing *in vitro* antiarthritic activities of formulation prepared from ethanolic extracts of *Cardiospermum helicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata* and *Vitex negundo* at 125, 250 and 500 µg/ml concentrations. Each value represents means ± SD (n=3).

Sl. No	*In vitro* antiarthritic activities	Acetyl Salicylic acid (50 µg/ml)	Formulation (All plant extracts added in equal ratio) (125 µg/ml)	Formulation (All plant extracts added in equal ratio) (250 µg/ml)	Formulation (All plant extracts added in equal ratio) (500 µg/ml)
1	Protein Denaturation inhibition (%)	98.88±0.82	89.54±0.89	93.21±0.83	96.24±0.96
2	Membrane Stabilitization (%)	94.11±0.79	87.24±0.91	90.67±0.76	93.32±0.86
3	Proteinase inhibition (%)	97.5±0.69	88.91±0.93	92.62±0.94	97.02±0.78

4. Conclusion

Arthritis stands as one of the commonest health troubles worldwide especially in developing countries. Allopathic therapies developed and followed so far are often limited with efficacy and carry the risk of adverse effects. They are very costly too, to afford by the common people. Therefore, treating arthritis with plant - derived compounds that are accessible to everyone and do not require laborious pharmaceutical synthesis seems highly attractive. Therefore, we have made an attempt to collect reported antiarthritic plants from Dakshina Kannada, Karnataka by discussing with people who use it in traditional medicine for treating arthritis. Plants such as *Cardiospermum helicacabum, Kirganelia reticulata, Pongamia pinnata, Scoparia dulcis, Urena lobata* and *Vitex negundo* were collected and ethanolic extracts were prepared to test 3 in vitro antiarthritic experiments such as protein denaturation inhibition, membrane stabilitization capacity and proteinase inhibition assay. All the plants have shown excellent effects and interestingly the effect has increased when all the extracts were combined together to a formulation. The obtained results may be useful to the scientists and health professionals working in field of pharmacognosy and therapeutics to develop novel and evidence - based alternative medicine to cure incidence of arthritis. Further studies are required to predict the phytoconstituents responsible for the anti - arthritic activity.

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