Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(2), 777-782 | ISSN:2251-6727

Comparative *In-Vitro* Evaluation of Herbal Extracts for Synergistic Effects of Anti-Inflammatory and Antioxidant Activity

Upasana Thakur[1], Mahendra Singh Ashawat[2]

[1]Department of Pharmaceutical Chemistry,Laureate Institute of Pharmacy, Kathog,Kangra,(HP)

[2]Director-cum-Principal, Laureate Institute of Pharmacy, Kathog,Kangra,(HP)

(Received: 07 January 2024 Revised: 12 February 2024 Accepted: 06 March 2024)

KEYWORDS

In-vitro anti-inflammatory activity, *In-vitro* antioxidant activity, DPPH method, Protein Denaturation, Bovine serum albumin, Synergistic effects

ABSTRACT:

Introduction: In the current work, in-silico docking investigation for 60 benzopyran-3-carbonyl derivatives was conducted since benzopyran derivatives play a vital role in many different research disciplines. The anti-arthritic activity of polyherbal extract was evaluated by *in-vitro* anti-inflammatory and *in-vitro* antioxidant activity. The anti-inflammatory activity was investigated by protein denaturation method using Bovine Serum albumin. Hydroalcoholic extracts of plants were used for evaluation at different concentration using Diclofenac sodium as reference drug at 660nm by using UV spectrophotometer. The *in-vitro* anti-oxidant activity of different hydroalcoholic extracts were measured by DPPH method using ascorbic acid as standard drug at 517nm using UV spectrophotometer method.The results revealed that all the herbal extracts possessed significant anti-inflammatory and anti-oxidant activities. But the maximum inhibition was obtained with the extracts when used in combination. The polyherbal extract showed protein denaturation at 300 µg/ml concentration is 75.29412 % as compared to the standard drug diclofenac sodium, which showed 91.2156% inhibition. The polyherbal extract showed *in-vitro* anti-oxidant activity with inhibition of 60.37037 % and that of reference drug ascorbic acid 99.26852%.

1. Introduction

Rheumatoid arthritis is a long-lasting provocative ailment of joints that results in various deformities. Development of ailment results in joint pain and joint destruction which can lead to various deformities and significant disability [1]. Herbal remedies play imperative role in management of various ailments. Numerous herbs used in the management of arthritis conventionally [2]. The consumption of natural products for treating life-threatening diseases is important because they have not been shown toxic effects as compared to the synthetic drugs used in modern therapy [3]. More than one herb when mixed in one formulation is known as polyherbal formulation. It is based upon the fact that plants have different phytoconstituents which are responsible for different activities and combination of more than one herbs in a single formulation will show better activity as compared to individual extract [4]. Plants constituent a large group of substances including: phenols and flavonoids. These phenolic compounds have various biological properties like anti-inflammatory and antioxidant. Antioxidant plays a substantial role in management of inflammation. These antioxidants can act as a shield for protecting the well-being of human health by inhibiting the propagation of free radical reactions [5,6,7]. Phenolic compounds act due to their redox properties as they can act as free radical terminators, hydrogen donors, quenchers and chelators [8]. Vitex genus comprises of numerous species, where *Vitex negundo* is also a very potent member of family Verbenaceae. The plant being aromatic shrub, having leaves of the plant is five-foliated which is 2 to 5 meter in length and erect [9]. *Vitex negundo* found in tropical and temperate climates including: Asia, China, Indonesia and India. This shrub generally available in Himachal Pradesh and possess various pharmacological activities viz; anti-inflammatory, anti-leprotic, anti-arthritic, and many other properties [10,11]. The second plant, *Cuscuta reflexa* is a parasitic perennial herb that belongs to family Convolvulaceae. This plant found in South Asian countries like Pakistan, Nepal, India, Bagladesh.

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JCHR (2024) 14(2), 777-782 | ISSN:2251-6727

The plant is known to possess various pharmacological activities like anti-inflammatory, anticholinergic, anti-histaminic, anti-hypertensive activity [12]. The genus Murraya is rich in alkaloids; approximately 14 species of Murraya are available across the globe. Only two species of Murraya are available in India, out of which *Murraya koenigii* belonging to family Rutaceae has been used in this study. Leaves of *Murraya koenigii* are known to possess activities like antioxidant, hepatoprotective, anti-microbial, anti-fungal, anti-inflammatory and nephroprotective. Traditionally the leaves of the plant have been used from ancient times as analgesics, digestives, appetizers as a home remedy. [13]. All these herbs have several pharmacological activities and these three herbs have been used from ancient times traditionally for the management of pain. The present study investigates that whether these three herbs when used in combination will provide a synergistic effect for the management of arthritis as compared to the individual extract of the plant.

2. Material and Methods

Plant Material

The plant material, leaves of *Vitex negundo*, stems of *Cuscuta reflexa*, and leaves of *Murraya koenigii* were collected from the local region of Kangra, Himachal Pradesh in the month of September. The collected plant material was authenticated by botanist Dr. Madhava Chetty, Department of Botany, Shri Venkateswara University, Tirupati, Andhra Pradesh, India. The collected plant material washed with running water and then with distilled water to remove the dirt particles. Washed plant material then subjected to drying under shade for 90 hours. Dried plant materials were powdered into coarse powder with the help of an electric blender. The powdered plant material is then passed through sieve number 40 and kept in airtight containers for further use [14].

Drugs and Chemicals

All reagents or chemicals used were of analytical grade.

2,2-diphenyl-1 picrylhydrazyl (DPPH) radical, Bovine Serum Albumin, Phosphate Buffer, Sodium nitrite ($NaNO_2$), Sodium hydroxide (NaOH), anhydrous Sodium carbonate (Na_2CO_3), Sulphuric acid (H_2SO_4) and reference standards Ascorbic acid, Diclofenac sodium were purchased from Merck and Sigma Aldrich. All the solvents used were of analytical grade. Rotary vacuum evaporator (Buchi Switzerland) was used for recovery of solvents under reduced pressure. UV/Vis Spectrophotometer (Shimadzu Pvt. Ltd., India) was used for taking absorbance of test samples.

Extract of plant material

The dried powdered plant material i. e. leaves of *Vitex negundo*, stems of *Cuscuta reflexa* and *Murraya koenigii* were extracted with hydroalcoholic solvent 80% (Ethanol). About 100 g of powdered material is subjected to Soxhlation. The powdered material was with 80 % ethanol using Soxhlet apparatus (Borosil) for 72 hours at room temperature. Each solvent is subjected for extraction for 72 hours. After siphoning extract of each plant is subjected to concentrate on a water bath and dried by using vacuum rotary evaporator. Then percentage yield of the extract was calculated and stored in air tight containers in dessicator for further experimental evaluation [15].

Determination of *in-vitro* anti-arthriic activity (Bovine serum albumin method [16,17]

The reaction mixture consisting of different concentrations of hydroalcoholic extract of *Cuscuta reflexa, Murraya Koenigii* and *Vitex negundo* of different extracts (0.05 ml) of various concentrations were prepared (50, 100,150, 200, 250, 300µg/ml). Then 0.45 ml 0.5 % Bovine Serum Albumin were added in all the test tubes. The samples were incubated at 37° C for 20 min and then heated at 57℃ for 20 min. After cooling, 2.5 ml of Phosphate buffer were added to the test tubes. Then the absorbance was measured after cooling at 660 nm. Percentage of inhibition of protein Denaturation was calculated as:

$$\% \, \boldsymbol{Inhibition} = [\frac{(\boldsymbol{Ao} - \boldsymbol{A1})}{\boldsymbol{Ao}}] \times \mathbf{100}$$

Determination of *in-vitro* antioxidant activity (DPPH method) [18]

In-vitro antioxidant study was done with DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging method with slight modifications. The reaction mixture of different concentrations of hydroalcoholic extract of *Cuscuta reflexa, Murraya Koenigii* and *Vitex negundo* were prepared by taking 2 ml of each in different test tubes. Then in each test tube 4 ml of 0.004% of DPPH solution was added. Each sample solution was then diluted with ethanol. The samples were incubated for

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half an hour in dark at room temperature for the completion of reaction. Each test tube was observed for colour change from dark violet to different degree of discolouration depending upon the concentration of antioxidant. Ascorbic acid was used as standard and stock solution was prepared from which different concentrations were prepared and standard curve was obtained. Each sample was taken in triplicate.

Absorbance was measured at 517 nm in UV Spectroscopy. 4ml of DPPH solution was used as negative control and ethanol was used as blank. The decrease in the absorbance with sample addition is used for calculation of antioxidant activity.

The percent of inhibition is calculated by using following formula:

$$\% \, \boldsymbol{Inhibition} = [\frac{(\boldsymbol{Ao} - \boldsymbol{A1})}{\boldsymbol{Ao}}] \times \mathbf{100}$$

Where, Ao = Absorbance of control, A1= Absorbance of sample.

3. Result and discussion

The *in-vitro* anti-arthritic activity of hydroalcoholic extracts of whole plant of *Vitex negundo*, *Cuscuta reflexa*, leaves of *Murraya koenigii* were evaluated by denaturation of proteins using Bovine serum albumin method. These extracts provided significant protection against denaturation of proteins at different concentration. Production of auto-antigens in certain rheumatic diseases may be due to *in-vivo* denaturation of proteins. Denaturation of proteins involves alteration in bonding viz., hydrogen, hydrophobic, electrostatic and disulphide bonds. Numerous anti-inflammatory drugs have shown dose-dependent ability to inhibit thermally induced protein denaturation [19,20,21]. Herbal extracts possess activity against denaturation of proteins. From these research findings these extracts were reported of possessing chemical constituents from different chemical classes like alkaloids, flavonoids, tannins, phenolic acids etc. These chemical classes known to provide protection against denaturation of proteins [22].

Current findings demonstrated that extracts when used alone have lower inhibitory effect with lower IC_{50} values viz; *Vitex negundo* (LI01): 19.9678, *Cuscuta reflexa* (LI02): 22.54512, *Murraya koenigii* (LI03): 19.43028 while when these extracts were used in combination showed more inhibition that is Polyherbal extract (LI04): 24.98545 with comparable IC_{50} value of standard drug Diclofenac sodium: 35.21803(Table no.1). Studies suggested that percent inhibitory effects of extract were increased as the concentration of the extracts were increased.

Table.1: Percent Inhibition of protein denaturation (Bovine serum albumin) of different extract [LI01; *Vitex neundo*, LI02 CR; *Cuscuta reflexa*, LI03 MK; *Murraya koenigii*, LI04; Polyherbal extract]

Sr. No.	Conc.(µg/ml)	Standard	LI01	LI02	LI03	LI04
1.	50	70.33333	60.82353	55.68627	50.72549	64.35294
2.	100	72.19608	62.78431	58.72549	51.70588	65.58824
3.	150	75.23529	64.7451	62.7451	54.7451	67.54902
4.	200	77.09804	66.5098	65.58824	55.82353	69.41176
5.	250	87.09804	67.39216	68.52941	57.19608	71.47059
6.	300	91.21569	71.40196	70.4902	58.47059	75.29412
	IC_{50}	35.21803	19.9678	22.54512	19.43028	24.98545

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JCHR (2024) 14(2), 777-782 | ISSN:2251-6727

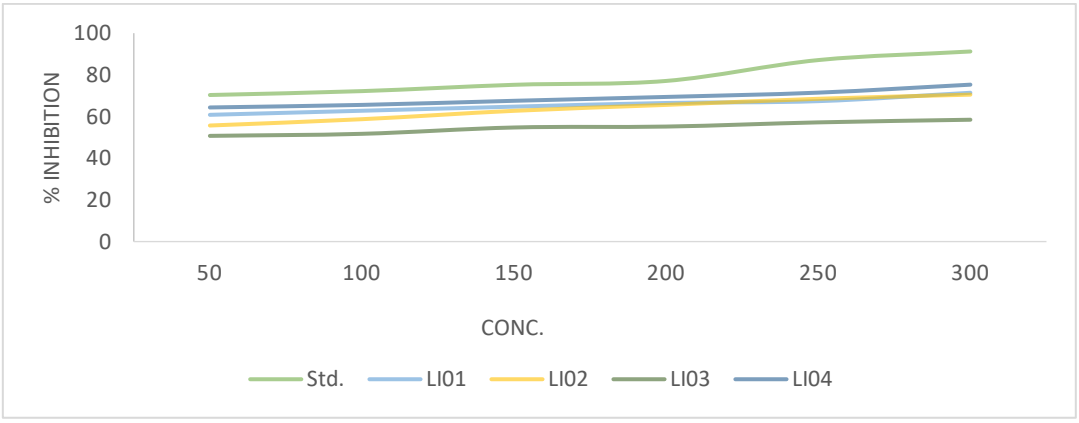

Fig. 1: Percent inhibition vs. concentration of protein denaturation of different extracts [LI01; *Vitex neundo*, LI02 CR; Cuscuta reflexa, LI03 MK; Murraya koenigii, LI04; Polyherbal extract

The effect of antioxidants is due to DPPH radical scavenging activity which is due to its hydrogen donating ability. Stable radical species are often used for the evaluation of general radical scavenging activities of antioxidants. DPPH radical scavenging activity, the inhibitory concentration 50% (IC_{50}) value was found to be, *Vitex negundo*: 158.8221 (LI 01), *Cuscuta reflexa* (LI02): 259.1615, *Murraya koenigii* (LI03): 206.4701 while when these extracts were used in combination showed more inhibition that is Polyherbal extract (LI04): 139.9601 with comparable IC_{50} value of standard drug Ascorbic acid: 101.6602 (Table no.2).The percent Radical Scavenging Activity of herbal extracts at maximum concentration showed results; *Vitex negundo*: 55.00010; *Cuscuta reflexa*: 42.777778; *Murraya koenigii*: 37.03704; Polyherbal Extract: 60.37037 and standard drug ascorbic acid: 99.26852. The results obtained in the present study clearly demonstrates that the polyherbal extract can effectively scavenge various ROS/free radicals under in-vitro conditions. This may be due to the number of stable oxidised products that it can form after oxidation or radical scavenging. Further studies, on the use of above plants for their antioxidant role in various systems may provide natural antioxidants. Results suggested that the percentage radical scavenging activity of herbal extracts increases as the concentration of the of the sample increases. The inhibitory concentration 50% (IC_{50}) value of standard ascorbic acid was found to be comparable results with the polyherbal extracts. The % RSA of Vitex negundo extract has more values as compared to the other extracts.

Table.2: Percent radical scavenging activity vs. Concentration (µg/ml) of different extracts and standard drug ascorbic acid

Sr. No.	Conc.(µg/ml)	Standard	LI01	LI02	LI03	LI04
1.	0.1	51.57407	27.77778	26.11111	14.44444	37.5000
2.	0.2	58.33333	31.01852	28.88889	18.33333	42.59259
3.	0.4	69.62963	36.48148	32.59259	21.66667	50.0120
4.	0.6	77.31481	40.74074	35.46296	26.11111	54.44444
5.	0.8	85.55556	46.2963	38.42593	27.03704	58.51852
6.	1	99.26852	55.0001	42.77778	37.03704	60.37037
7.	IC_{50}	101.66	158.82	259.16	206.47	139.96

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JCHR (2024) 14(2), 777-782 | ISSN:2251-6727

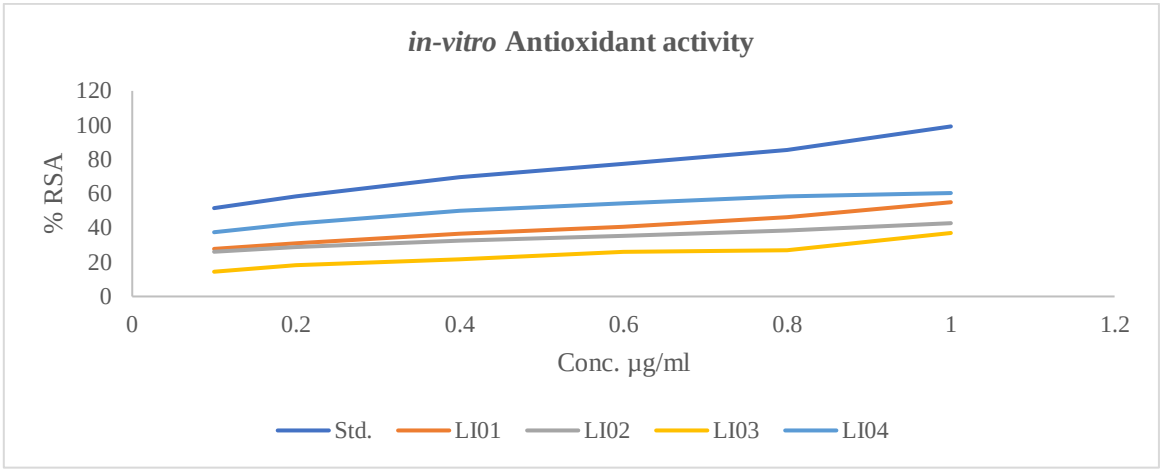

Fig. 2: Percent radical scavenging activity vs. Concentration (µg/ml) of different extracts and standard drug ascorbic acid

4. Conclusion

The present findings suggested that when these herbs used in combination can effectively scavenge the reactive oxygen species effectively under in -vitro conditions. These extracts also possess significant protection against denaturation of proteins in a dose dependent manner. Further studies warranted for the isolation and identification of individual phenolic compounds and also in-vivo studies are needed for understanding their mechanism of action to exploit as potent antioxidants for therapeutic applications.

Acknowledgment: We are thankful to the principal, management and colleagues for their support and guidance.

Conflict of Interest: The authors states that no conflict of interests

Financial Support: Self support

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JCHR (2024) 14(2), 777-782 | ISSN:2251-6727

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