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IN VITRO ANTIARTHRITIC, ANTIOXIDANT AND *IN VIVO* CYTOTOXIC ACTIVITY OF YOGARAJA GUGGULU

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ABSTRACT: Yogaraja guggulu a poly herbal formulation, consisting of 29 ingredients, has been used for the neurological, musculoskeletal disorders and in general practice, used for osteo arthritis. *In vitro* antiarthritic activity is done against pathogens which also include septic arthritis causing microorganisms. This study focused on determining antimicrobial efficacies of methanol (A), ethylacetate (B) and hexane extracts (C) of the *Yogaraja guggulu* by measuring the zone of inhibition. *In vitro* antioxidant activities were also assayed by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method. Antimicrobial activity was tested against the pathogenic bacteria: *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus megaterium*, *Klebsiella pneumonia*, *Enterobacter aerogenes*, *Enterococci bacteria*; Pathogenic fungi: *Candida albicans*, *Candida tropicalis*, *Candida bombi*, *Candida utilis* and *Trichophyton rubrum*. In these three extracts methanol extract (A) showed better zone of inhibition (19-50 mm) when compared with ethyl acetate (B) and hexane (C) (9-40 mm) and (9-45 mm) respectively, against the tested pathogens. DPPH antioxidant activity of all the three extracts showed potent radical scavenging activity in comparison with the positive control, ascorbic acid. The IC_{50} values are 424.89, 419.39, 413.60 and 394.56 ug/ml for A, B, C and ascorbic acid, respectively. The FRAP method also showed excellent antioxidant potential with methanol extract (A) (90.454 mg of Gallic acid equivalents/g) when compared with other extracts B (86.571 mg GAE/g) and C (82.472 mg GAE/g). *In vivo* Brine Shrimp Lethality Assay (BSLA) of the methanol (A), ethyl acetate (B) and hexane extracts (C) of the *Yogaraja guggulu* and correlate cytotoxicity results with known pharmacological activities of the plants. Novel cytotoxic, antitumor compounds can be isolated from potential plant sources through the assessment of cytotoxic activity against brine shrimps. Cytotoxicity was evaluated in terms of LC50 (lethality concentration). Ten nauplii were added into three replicates of each concentration of the test samples. After 24 hours the surviving brine shrimp larvae were counted and LC50 was assessed. Results showed that the extracts of methanol, ethyl acetate and hexane extracts were potent against the brine shrimp with LC50 values of 55, 10, and 100 ppm (µg/mL), respectively. It indicated that bioactive components are present in these plants that could be accounted for its pharmacological effects. The results validate the potent use of against septic arthritis, rheumatoid arthritis and skin diseases. The results are an evidence for the existence of synergism among the compounds of different ingredients and validate the use of Yogaraja guggulu for various ailments.

INTRODUCTION: Guggulu is an herbal remedy based on purified guggulipid in Ayurvedic medicine. Antiallergic, antibacterial and blood purifying properties are found in this herbal preparation [1].

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Guggulu is exudates obtained in the form of oleo gum resin from the stem of the plant *Commiphora mukul* belongs to Burseraceae family. It is known to have analgesic, anti-inflammatory activity etc. It is used in various Ayurvedic formulations. Yogaraja guggulu is a traditional formula designed to reduce excess vata in the system. It is particularly useful for accumulated vata in the joints and muscles, which may be indicated by cracking or popping joints, tics, spasms or tremors. Chronic accumulation may lead to such serious conditions as rheumatism and arthritis. Yogaraja guggulu contains a synergistic blend of detoxifying herbs, including triphala, chitrak and vidanga that work

in conjunction with guggulu to remove excess vata from the joints as well as the nerves and muscles. [2]

Although Yogaraja guggulu is best known as an anti-arthritic herbal supplement composed of a number of herbs. Yet this is also known for purifying and rejuvenating the body and mind. This is also used to treat inflammatory conditions such as osteoporosis, bone density, arthritis, especially rheumatism and gout. Yogaraja is a powerful anti-oxidant, and stimulates the immune system's white blood cells (WBC's).[3] Other indications include menstrual disorders and digestive disturbances, and it is a general tonic to the eyes, hair, and the nervous system, skin problems, genito-urinary disorders, as well as obesity, and fat, weight reduction.[4] Also recent research has shown Guggulu is one of the most powerful cholesterol-lowering agents known, also lowering the triglycerides.[5] The herbs also have antispasmodic carminatives, with laxative and detoxifying properties.

Septic arthritis is the purulent invasion of a joint by an infectious agent which produces arthritis. The infection may also spread to other parts of the body. However bacteria cause the most damage to the joint and they cause septic arthritis. Septic arthritis is more commonly seen in children and in elderly adults. Septic arthritis if not treated quickly can result in significant permanent damage to the joint and disability for the patient. Septic arthritis can be caused by bacteria, viruses, and fungi. The most common causes of septic arthritis are bacteria. According to the National Institutes of Health (NIH) the most common cause of septic arthritis is *Staphylococcus aureus*. Chronic septic arthritis is caused by organisms such as *Mycobacterium tuberculosis* and *Candida albicans*. *E. coli*, *Proteus, Serratia, Neisseria gonorrhoeae* are also common causatives; *Klebsiella pneumonia, Enterobacter aerogenosa*, *Enterococcus* species are also known to cause septic arthritis.

Some of the traditional medicine involves the use of crude plant extracts which may contain an extensive diversity of molecules, often with indefinite biological effects. However, most of the available information regarding the medicinal potential of these plants is not provided with credible scientific data. For this reason, several researches have been conducted to determine the toxicity of medicinal plants. A general bioassay that appears capable of detecting a broad spectrum of bioactivity present in plant crude extracts is the Brine Shrimp (*Artemia* sp.) Lethality Assay (BSLA). [6] BSLA is used as an indicator for general toxicity and also as a guide for the detection of antitumor and pesticidal compounds. The low cost and ease of performing the assay and the commercial availability of inexpensive brine shrimp eggs makes BSLA a very useful bench top method.[7] This assay has been noted as a useful tool for the isolation of bioactive compounds from plant extracts.

The present work is undertaken to test yogaraja guggulu for its efficiency in treating septic arthritis, skin diseases and rheumatoid arthritis; and validate the traditional practice of using this Ayurvedic medicine. However, the current work emphasize on the synergistic property of a polyherbal formulation, Yogaraja guggulu by testing its antimicrobial activity, antioxidant and cytotoxic activity. *In vivo* test for their cytotoxic effect against the brine shrimp nauplii and relate toxicity results with their known ethno-pharmacological activities. *In vivo* lethality test has been successfully used as a preliminary study of cytotoxic and antitumor agents. Thus, the findings of this present work would give baseline information on the most promising plant species that could be use as a basis for the development of new tools of great therapeutic importance. Yogaraja guggulu was selected for this study based on its frequent usage in traditional medicine for treating both arthritis as well as skin diseases.

MATERIALS AND METHODS:

Collection of plant material:

The poly herbal formulation of Yogaraja guggulu was procured from an Ayurveda nilayam, by an Ayurvedic physician, which was prepared using 29 ingredients [8] based on traditional methods in accordance with the procedures given in classical texts like *Bhaishajya Ratnavali* and being used for the patients with arthritis pains. Yogaraja guggulu thus obtained was extracted separately using methanol, ethyl acetate and hexane solvents as described below.

Solvent Extraction:

The sample was brought to the laboratory; 100 g of the formulation was extracted successively in methanol (A), ethyl acetate (B) and hexane (C) by using 250 ml of each solvent for soaking. Maceration was carried out in each solvent for four days at room temperature (35 ± 2 °C) by keeping them in orbital shaker. The extracts were respectively concentrated *in vacuo* at 40 °C using a rota vapor. The crude extracts thus obtained were preserved in freezer at -20°C until use.

Test microorganisms and microbe culture:

The microorganisms that are known to cause septic arthritis and skin diseases are selected for the present study. Bacterial and fungal strains used in the current work include clinical and pure isolates of seven bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus megaterium*, *Klebsiella pneumonia*, *Enterobacter aerogenosa*, *Enterococci species*; and five fungal isolates such as *Candida albicans*, *Candida tropicalis*, *Candida bombii*, *Candida utilis* and *Trichophyton rubrum*. The strains were obtained from the Department of Microbiology, Andhra Medical College, Visakhapatnam. Nutrient agar (NA) and Potato dextrose agar (PDA) were used for the growth of bacteria and fungi, respectively. All the cultures were tested for their purity. Pure cultures thus obtained were kept on respective agar slants at 4 °C until needed. They were sub-cultured once in every month. Each inoculum was prepared by inoculating the stock culture into freshly prepared media. All the bacterial strains were incubated at 37 °C for 24 h and fungi at 27 °C for 48h. The test organisms were grown overnight in respective broth media.

Determination of Antimicrobial Activity:

Antimicrobial activity of organic extracts of the plant samples were evaluated by the paper disc diffusion method. [9] For determination of antibacterial activity, overnight grown bacterial cultures were adjusted to 0.5 McFarland turbidity standards. For the determination of antifungal activity, all the fungal isolates were first adjusted to the concentration of 106cfu/ml. The bacterial and the fungal broth cultures of 100µl each were inoculated onto Nutrient Agar and Potato dextrose Agar plates respectively through spread plate method. Firstly stock solutions (100µg/ml) of each solvent extract were prepared separately. Sterile filter paper (Whatman filter paper No.1) discs of diameter 9 mm were prepared and 10 µl of each extract dilutions were impregnated onto the discs and carefully placed at the centre of the previously seeded plates with 0.5 McFarland and 10^6 cfu/ml cultures of bacteria and fungi respectively with sterile forceps. Disc with solvent alone was served as control. Rifampicin (10 µg/ml) for bacteria and Griseofulvin (10 µg/ml) for fungi was used as standard antimicrobials for comparison.

Bacterial cultures plates were then incubated at 37°C for 24 h while the fungal cultures are incubated at (25 ± 2 °C) for 48h. Antimicrobial activity was determined by measurement of zone of inhibition of growth around each paper disc (mm). For each extract (test) three replicate trials were conducted against each organism. Each zone of inhibition was measured with a ruler and compared with standard. [10]

Determination of *in vitro* antioxidant activity assay:

***In vitro* antioxidant activity by DPPH assay:**

DPPH method is based on the reduction of alcoholic DPPH solution (dark blue in colour) in the presence of a hydrogen donating antioxidant converted to the non radical form of yellow colored diphenyl-picrylhydrazine. 4mg of DPPH was dissolved in 100ml of ethanol and kept it overnight in dark place for the generation of DPPH radical. The scavenging activity for DPPH free radicals was measured according to the procedure described by Braca et al., 2003.[11] An aliquot of 3ml of 0.004% DPPH solution in ethanol and 0.1ml of plant extract at various concentrations were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30min. decolorization of DPPH was determined by measuring the absorbance at 517nm. A control was prepared using 0.1ml of respective vehicle in the place of plant extract or ascorbic acid.

***In vitro* antioxidant activity by FRAP assay:**

The ferric reducing antioxidant power [12] property of the extract was determined by taking 1 ml of different dilutions of standard solutions of gallic acid (10-100 µg/ml) or methanolic extract that had

been adjusted to come under the linearity range (500 µg/ml), placed in 10-ml volumetric flasks and mixed with 2.5 ml of potassium buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50°C for 20 min and 2.5 ml of 10% trichloroacetic acid was added to the mixture to stop the reaction.

To the 2.5 ml of the above solution, 2.5 ml of distilled water was added and then 0.5 ml of 0.1% $FeCl_3$ was added and allowed to stand for 30 min before measuring the absorbance at 593 nm. The absorbance obtained was converted to gallic acid equivalents in mg/g of dry material (GAE/g) using a gallic acid standard curve. [13]

Brine Shrimp Lethality Assay (BSLA):

Brine shrimp eggs were obtained from the department of Zoology, Dr. V.S. Krishna Govt Autonomous College for the research work. Filtered, artificial seawater was prepared by dissolving 38 g of sea salt in 1 liter of distilled water for hatching the shrimp eggs. The seawater was put in a small plastic container (hatching chamber) with a partition for dark (covered) and light areas. Shrimp eggs were added into the dark side of the chamber while the lamp above the other side (light) will attract the hatched shrimp.

Two days were allowed for the shrimp to hatch and mature as nauplii (larva). After two days, when the shrimp larvae are ready, 4 mL of the artificial seawater was added to each test tube and 10 brine shrimps were introduced into each tube.

Thus, there were a total of 30 shrimps per dilution. Then the volume was adjusted with artificial seawater up to 5 mL per test tube. The test tubes were left uncovered under the lamp. The number of surviving shrimps were counted and recorded after 24 hours. Using probit analysis, the lethality concentration (LC50) was assessed at 95% confidence intervals. LC50 of less than 100 ppm was considered as potent (active). [14] As mentioned by Meyer and others, LC50 value of less than 1000 µg/mL is toxic while LC50 value of greater than 1000 µg/mL is non-toxic. The percentage mortality (%M) was also calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100%. This is to ensure that the death (mortality) of the nauplii is attributed to the bioactive compounds present in the plant extracts.

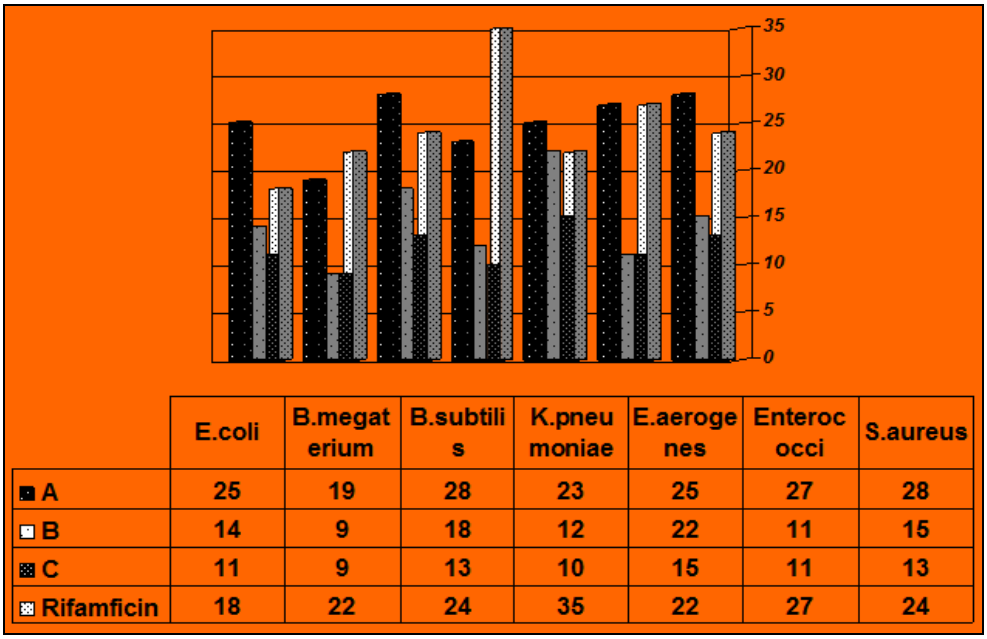
RESULTS:

The present study was conducted to investigate the antimicrobial, antioxidant and Cytotoxic properties of a poly herbal formulation, of Yogaraja guggulu which is been traditionally used for obesity, body pain, skin disorders, neurological and musculo skeletal problems. [8] In the present study, an attempt has been made to correlate traditional herbal medicinal knowledge held by the Indian native people with modern scientific laboratory-based assay. Antimicrobial screening of different solvent extracts of yogaraja guggulu are shown in (**Fig. 1 & 2**).

The present study reveals that the three solvent extracts showed potent antibacterial and antifungal activity against all reference microbial strains. Among the tested solvent extracts, methanol extract exhibited highest antibacterial activity with 19-28 mm of zone of inhibition against all the reference strains (**Fig.1**), which was nearly equal to activity of standard antibiotic rifampicin (18-35 mm).

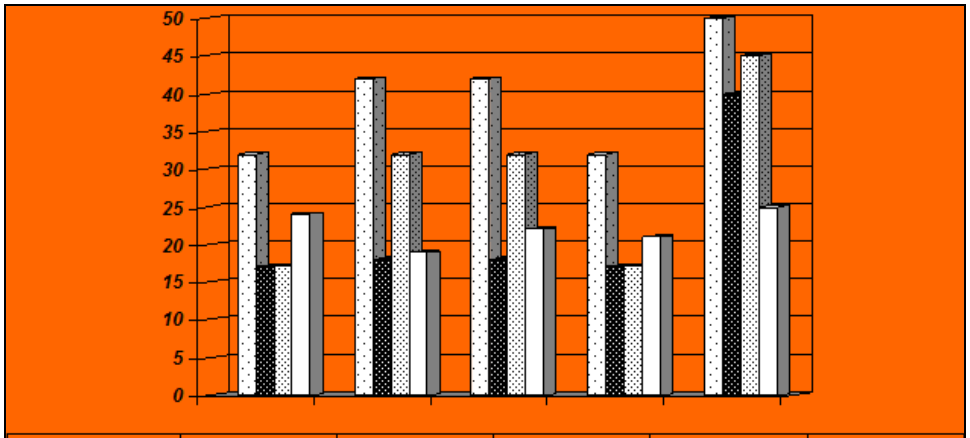
The next highest activity was showed by ethyl acetate extract (9-22 mm) followed by hexane extract (9-15 mm). Methanol extract even exhibited highest antifungal activity (32-50 mm) which is comparatively higher than the standard antibiotic griseofulvin (19-25 mm) (**Fig. 2**). Next highest antifungal activity was observed in hexane extract (17-45 mm) followed by ethyl acetate extract (17-40 mm).

As it is best to correlate the results based on different assays to get a clear picture of the antioxidant activity of complex samples, *in vitro* antioxidant activity of three solvent extracts of Yogaraja guggulu was assessed based on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and the ferric reducing antioxidant power (FRAP). DPPH antioxidant activity of all the three extracts showed potent radical scavenging activity. The results were compared with the positive control, ascorbic acid (**Table 1 and Fig. 3**).



	E.coli	B.megaterium	B.subtilis	K.pneumoniae	E.aerogenes	Enterococci	S.aureus
A	25	19	28	23	25	27	28
B	14	9	18	12	22	11	15
C	11	9	13	10	15	11	13
Rifamficin	18	22	24	35	22	27	24

FIG.1: BAR CHART SHOWING RESULTS OF ANTIBACTERIAL SUSCEPTIBILITY OF TEST ORGANISMS TO METHANOLIC (A), ETHYL ACETATE (B), HEXANE (C) EXTRATS OF YOGARAJA GUGGULU AND RIFAMPICIN SAMPLES



3-D Column 1	C.albicans	C.bombii	C.utilis	C.tropicalis	T.rubrum
A	32	42	42	32	50
B	17	18	18	17	40
C	17	32	32	17	45
Griseofulvin	24	19	22	21	25

FIG.2: BAR CHART SHOWING RESULTS OF ANTIBACTERIAL SUSCEPTIBILITY OF TEST ORGANISMS TO METHANOLIC (A), ETHYL ACETATE (B), HEXANE (C) EXTRATS OF YOGARAJA GUGGULU AND RIFAMPICIN SAMPLES

TABLE 1: CONCENTRATION DEPENDENT PERCENT INHIBITION OF DPPH RADICAL OF METHANOL (A), ETHYL ACETATE (B), HEXANE (C) EXTRACTS OF YOGARAJA *GUGGULU* AND ASCORBIC ACID

conc.(ug/ml)	A	B	C	Ascorbic acid
10.0	1.282	1.106	1.175	1.196
25.0	5.089	5.495	6.991	4.225
50.0	11.482	11.059	12.769	12.362
100.0	19.046	19.679	20.658	24.479
250.0	30.916	30.153	32.658	40.256
500.0	43.692	42.572	45.867	58.842
750.0	65.683	66.876	69.551	64.673
1000.0	76.645	75.346	79.021	80.724

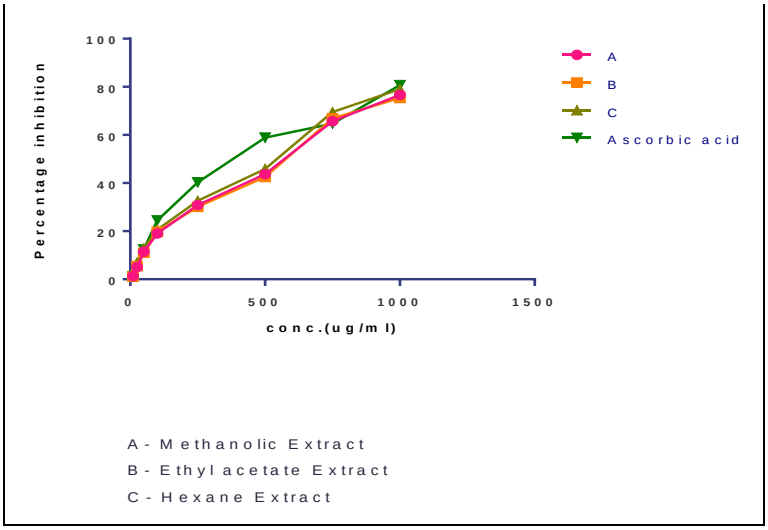


FIG. 3: CONCENTRATION DEPENDENT PERCENT INHIBITIONS OF DPPH RADICAL BY METHANOL EXTRACT (A), ETHYL ACETATE EXTRACTS (B), HEXANE EXTRACT (C) OF YOGARAJA GUGGULU AND ASCORBIC ACID.

Among the extracts tested hexane showed maximum percentage of inhibition followed by methanol and ethyl acetate. The IC_{50} values are 424.89, 419.39, 413.60 and 394.56 ug/ml for methanol, ethyl acetate, hexane and ascorbic acid, respectively (**Table 2**).

TABLE 2: *IN-VITRO* 50% INHIBITION CONCENTRATION (IC_{50}) OF METHANOL (A), ETHYL ACETATE (B), HEXANE (C) EXTRACTS OF *YOGARAJA GUGGULU* AND ASCORBIC ACID DPPH FREE RADICALS

Name of the fraction	IC_{50} value for DPPH radical (ug/ml)
A	424.89
B	419.39
C	413.60
Ascorbic acid	394.56

FRAP method also showed excellent antioxidant potential the three extracts. Maximum activity is exhibited by methanol extract with 90.454 mg of Gallic acid equivalents/g when compared with other extracts ethyl acetate (86.571 mg GAE/g) and hexane (82.472 mg GAE/g).

The crude extracts of the three test samples tested showed good brine shrimp larvicidal activity. The lethality concentration (LC50) of *methanol, ethyl acetate and hexane* extract of Yogaraja guggulu were 10 ppm (**Table 1**). The degree of lethality was directly proportional to the concentration of the extract. Maximum mortalities (100%) were observed at a concentration of 1000 ppm in Mehtanol, Ethyl acetate and Hexane extracts of Yogaraja guggulu. Based on the results, the brine shrimp lethality of the three solvents crude extract of Yogaraja guggulu were found to be concentration-dependent.

The observed lethality of the crude extracts to brine shrimps indicated the presence of potent cytotoxic and probably antitumor components of these plants. According to Meyer et al. [15], crude plant extract is toxic (active) if it has an LC50 value of less than 1000 µg/mL while non-toxic (inactive) if it is greater than 1000 µg/mL.

TABLE 3: THE NUMBER OF SHRIMP NAUPLII THAT SURVIVED AFTER TREATING WITH THE METHANOL, ETHYL ACETATE AND HEXANE EXTRACTS OF YOGARAJA GUGGULU THE PERCENTAGE MORTALITY.

Plant Extracts	Concentration (ppm or µg/mL)	Number of Surviving Nauplii After 24 h			Total Number of Survivors	% Mortality
		T1	T2	T3		
Methanol	1	6	7	7	20	35%
Extract (A)	10	4	6	5	16	50%
	100	0	0	0	0	100%
	1000	0	0	0	0	100%
Ethyl acetate	1	6	7	8	21	31%
Extract (B)	10	5	5	6	15	47%
	100	0	0	0	0	100%
	1000	0	0	0	0	100%

Hexane	1	7	7	8	22	27%
Extract (C)	10	5	6	5	16	47%
	100	0	0	0	0	100%
	1000	0	0	0	0	100%

The results showed that the crude extracts of Yogaraja guggulu were potent or active against brine shrimps where *Methanol extract* was the most active at 10 ppm. Both ethyl acetate and hexane extracts of Yogaraja guggulu also shows potent or active against brine shrimps at 10 ppm in this present study. Fatope et al. [16] screened for the activity of the Yogaraja guggulu on brine shrimp larvae and was able to isolate oleanonic acid, lantadene A, and oleanolic acid which exhibited significant toxicity on the larvae.

Moreover, they also noted that crude extracts inhibit the development of the phytopathogenic fungus *Cladosporium sphaerospermum*. This could be attributed to the presence of cytotoxic substances like camaraside and lantanoside. Previous related studies showed that the essential oil from this plant is active against fungi and bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The major essential oils from Yogaraja guggulu are sesquiterpenes which were cytotoxic to V79 mammalian cells and also to *Artemia salina*, showing 50% lethal concentration (LC50) values from 0.23 µg/mL. There *in vitro* data suggested that essential oil of this plant may also be effective in treating yeast infection. In the other hand, some studies have shown that *crude* extracts exhibited selective cytotoxicity against several cancer cell lines.

In one study, the extracts were noted as having an anti-proliferative activity against the normal mouse fibroblast cells. Furthermore, the crude extract of samples showed an anti-proliferative activity against Hep-2 cells obtained from human epithelioma of the larynx. The results further confirmed the good activity of the poly herbal formulation, Yogaraja guggulu against septic arthritis, skin diseases also proved to have potent antioxidant activity and cytotoxic activity.

DISCUSSION: The results in the current study clearly reveal the therapeutic value of the yogaraja guggulu. The reports on antimicrobial activity state that the poly herbal formulation is potent against both septic arthritis as well as skin disease causing microbes. This activity might be due to the presence of ample number of active constituents that either lessen or prevent the growth of microbial colonies.

The phytochemical constituents in the ingredients of yogaraja guggulu were more of tannins, alkaloids, terpenes, flavonoids. Glycosides and steroids were also found in few ingredients. [8] The presence of different secondary metabolites, especially those known to be responsible for antimicrobial activity, was confirmed and these metabolites in plants have been linked to the potent antimicrobial activity [17,18] . This is explained by the fact that the secondary metabolites identified in the extracts are known to have combinational antimicrobial properties. Among the solvent extracts tested methanol extract registered maximum activity, as more or less all of the antimicrobial compounds known from plants are saturated organic or aromatic compounds, so might have been easily extracted by the polar organic solvent, methanol.

Free radicals are implicated in chronic inflammatory diseases including rheumatoid arthritis. Free radicals have been implicated as mediators of tissue damage and play an important role in the severity of rheumatoid arthritis and patients usually suffer high oxidative stress [19,20] . Antioxidants may have a possible role in improving inflammatory condition in rheumatoid arthritis patients [21]. So, the present study also aimed to identify *in vitro* antioxidant activity of different solvent extracts of yogaraja guggulu in order to correlate with its anti-inflammatory activity.

All the three extracts have showed potent antioxidant activity in DPPH assay which might be due to the presence of phenolic compounds like tannins and flavonoids in the ingredients of yogaraja guggulu whereas methanol extract is more potent in FRAP method. These differences might be due to their different antioxidant mechanisms or variations in their ability to scavenge free radicals.

Different methods used for antioxidant activity evaluation can give varying results based on the specific free radical being used as a reactant and it is likely that each of these methods determine rather a different profile of antioxidant compounds [22]. Methods like FRAP measure only the hydrophilic antioxidants, while others like DPPH detect merely those soluble in organic solvents, particularly alcohols [23].

The secondary metabolites from plants are good sources for combination therapy. The therapeutic indications of medicinal plant-based extracts are in most cases, empirical, and practitioners of phytotherapy intuitively believe that a total extract acts better than an equivalent dose of an isolated substance [24].

CONCLUSION: The obtained results might be considered sufficient to further studies for the isolation and identification of the active principles and to evaluate the possible synergism among extract components for their antioxidant and antimicrobial activity. The crude extract of three different solvent samples exhibited cytotoxic activity against the brine shrimp and considered as containing active or potent components. This is because their LC50 values are less than 1000 ppm or µg/mL. The ethno-pharmacological activities of these plant species are due to the different bioactive compounds present in these plants. Although, BSLA is inadequate in determining the mechanism of action of the bioactive substances in the crude sample, it is very useful by providing a preliminary screen that can be supported by a more specific bioassay, once the active compound has been isolated. Thus, some useful drugs of therapeutic importance may develop out of the research work.

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