



Research Journal of
**Medicinal
Plant**

ISSN 1819-3455



Academic
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***In vitro* Validation and Phyto-constituent Analysis of Turmeric Extract: An Ethnological Alternative for Eye Treatment**

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ABSTRACT

The essential oil from the rhizomes of Roma cultivar of turmeric (*Curcuma longa*) from Orissa was examined for its antimicrobial activity against the pathogens causing eye infections. The purpose of this study was to authenticate the use of turmeric rhizome oil against eye infections so as to giving an approach to formulate turmeric rhizome oil as potential eye drop in place of traditional antibiotics after undertaking its *in vivo* pharmacological studies. Essential oil from rhizomes of Roma cultivar obtained by hydrodistillation extraction method using clenvevger apparatus. The antimicrobial effects of oil towards *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger* were tested by Inhibition Zone Diameter (IZD) test to screen the antimicrobial activity, Minimum Inhibitory Concentration (MIC) test and Minimum Killing Time (MKT) test to determine the minimum concentration of oil and minimum time required to kill the pathogens. Oil showed very good activity against all four microbial strains used at concentration of 10 μL except *Pseudomonas* sp. Very low concentration of 1.95 $\mu\text{L mL}^{-1}$ oil was needed to inhibit the growth of most highly infecting pathogen *Staphylococcus aureus* within 15 min of its exposure in comparison to other microbial strains. High turmerone content (49.76%) of elite turmeric cultivar Roma released from Orissa (India) might be assigned to be responsible for such excellent anti microbial activity against the tested pathogens.

Key words: Turmeric, antimicrobial activity, phyto constituents, inhibition zone diameter, eye ailments

INTRODUCTION

Ailments related to eye continue to be a major health problem worldwide (Lowy, 1998). Severe eye infections like blepharo conjunctivities, corneal ulcers, abscesses, styes, dacryocystitis, periorbital-celluties, orbital cellulitis and blebs are mainly caused by *Staphylococcus aureus*, a normal flora (Groden *et al.*, 1991) and *Pseudomonas aeruginosa*, an opportunistic human pathogen (Stapleton *et al.*, 1995). *Candida albicans* and *Aspergillus niger* are other most common cause of endogenous endophthalmitis, leading to scarring of the chorioretina and blindness (Anonymous, 1979). The present treatment of choice for these pathogens is antibiotics which brings about severe side effects like hypersensitivity reactions, gastric disturbances, ototoxicity and nephrotoxicity (Cosgrove *et al.*, 2009) and incites resistance against these pathogens (Poole, 2004). On the other hand, there exist many advantages in using antimicrobials obtained from medicinal plants such as fewer side effects, relatively less expensive, better patient tolerance, acceptance due to long

history of use and being renewable in nature (Kim, 2005; Prabu *et al.*, 2006). Ethno botanical search and review of ancient traditional medicinal scripts on palm leaves from different districts of Orissa accentuates the use of turmeric for treating eye infections by tribal community Kond, Kui and Dongria of Kandhamal district and Bondas, Bhunia, Paroja and Dora of Koraput district (www.shroong.com, www.infinityfoundation.com). Other ethnological groups like common people of remote villages, traditional healers and as well as qualified medicinal practitioners of Ayurved, Naturopathy, Unani and Sidha have been found treating several eye ailments using turmeric nationwide even till today. Drops of strained water extract, obtained from boiling turmeric powder few minutes in water have been used for treatments of eye problems by these tribal groups in few districts of Orissa. Turmeric has got enormous ethnological use in treatment of several diseases in different states of India (Ammon *et al.*, 1992) including Orissa (www.shroong.com, www.infinityfoundation.com) and also in many Asian and African countries like China (Araujo and Leon, 2001), Nepal (Eigner and Scholz, 1999), Thailand (Amphawan *et al.*, 1995; Mahady *et al.*, 2002), Nigeria (Usman *et al.*, 2009) and Brazil (Araujo and Leon, 2001) for its range of medicinal properties. It has been cultivated at a commercial scale in all these states for its use in medicine, spice and flavoring agent. Orissa has been famed as the 2nd largest producer of turmeric in India and is the home state for releasing of many promising turmeric cultivars Surama, Roma, Ranga etc. possessing high rhizome and/or drug yielding potential (Ravindran *et al.*, 2007). *In vitro* and *in vivo* experimental verification of ethnological usage of turmeric has been done to establish its medicinal properties like anti inflammatory, antioxidant (Prakash *et al.*, 2003), photo receptor, wound healing etc. Jorge (2004) including its anti infecting potential (Srimal and Dhawan, 1985) by various workers by using essential oil or other chemical extracts and its derived compounds (Chattopadhyay *et al.*, 2004). Some of the properties are well documented and validated by pharmacological and clinical trials, while many remains still to be validated (Duke, 2003). Review of literature does not illustrate any single report where a concerted effort was taken to find the effect of turmeric rhizome essential oil on inhibition of group of microorganisms responsible for causing various eye infections e.g., *Staphylococcus aureus*, *Pseudomonas aerogenosa*, *Candida albicans* and *Aspergillus niger*. Phytoconstituents of essential oil of medicinal plants are established to be responsible for the biological activity against microbial pathogens (Kaur and Arora, 2009). Therefore an attempt is taken to authenticate the traditional use of turmeric by ethnological groups against common diseases related to eye, in terms of evaluating the effectiveness of rhizome essential oil collected from a high ar-turmeorne containing elite turmeric cultivar Roma in inhibiting the growth of specific eye infection causing pathogens. Validation of ethnological use of turmeric (cv. roma) essential oil *in vitro* is done through performing all the basic tests like; determination of IZD (Inhibition Zone Diameter), MIC (minimum inhibitory concentration), bactericidal and fungicidal effect, MKT (Minimum killing time) so as to enable an opening to formulate turmeric oil as potential eye drop in place of traditional antibiotics after undertaking it's *in vivo* pharmacological studies.

MATERIALS AND METHODS

Plant material: The rhizome of *Curcuma longa* (cv. roma) were collected from the High Altitude Research Station Pottangi, Orissa and grown in medicinal plant garden of Centre of Biotechnology, Bhubaneswar, Orissa (India). Fresh leaves and rhizomes on harvest were collected, washed under running tap water and were used immediately to extract the essential oils.

Microorganism and media: The test organisms used in this study were *Staphylococcus aureus* (MTCC-3160), *Pseudomonas aerogenosa* (MTCC-424), *Candida albicans* (MTCC-183) and *Aspergillus niger* (MTCC-281) obtained from the Microbial Type Culture Collection, Chandigarh, India. All the strains maintained in recommended media purchased from Hi-Media India private Ltd., Mumbai. This research project was conducted from 2nd December 2009 to 2nd June 2010.

EXTRACTION OF ESSENTIAL OILS

The rhizome of *Curcuma longa* (cv. roma) were collected from the High Altitude Research Station, Pottangi, Orissa and grown in medicinal plant garden of Centre of Biotechnology, Bhubaneswar, Orissa (India). Fresh rhizomes on harvest were collected, washed under running tap water and were used immediately to extract the essential oils. The fresh rhizomes of turmeric were washed to remove soil, peeled and sliced. Sliced rhizomes of fresh turmeric (100 g) were mixed with distilled water. The essential oil was extracted by hydro-distillation using a Clevenger's apparatus following the method of Guenther (1948) out at room temperature. A flask containing the homogenate was heated for 6-10 h and the condensed vapor was separated throughout an auto-oil/water separator. The oil present at the upper most layers was collected in the ependroff tube. Each essential oil extraction was run in duplicate to evaluate the oil yield. The solubility of essential oil in water as well as other organic solvents was tested.

The total amount of oil in rhizome was calculated by following method. Yield percentage was recorded as dry weight basis.

$$\text{Rhizome oil \% yield (v/w) (dry weight)} = \frac{\text{Volume of essential oils (mL)}}{\text{Weight of raw materials}} \times 100\%$$

ANTIMICROBIAL ACTIVITY ASSAY OF ESSENTIAL OIL

The test organisms used in this study were *Staphylococcus aureus* (MTCC-3160), *Pseudomonas aerogenosa* (MTCC-424), *Candida albicans* (MTCC-183) and *Aspergillus niger* (MTCC-281) obtained from the Microbial Type Culture Collection, Chandigarh, India. All the strains maintained in recommended media were purchased from Hi-Media India private Ltd., Mumbai.

Determination of Inhibition Zone Diameter (IZD): Initial screening through inhibition zone diameter was determined by the disc diffusion method as described previously (Pattnaik *et al.*, 1995) with slight modifications. Briefly, Nutrient Agar (NA) and Potato Dextrose Agar (PDA) plates were swabbed with freshly grown cultures of the test pathogens by the help of a pre-sterilized cotton swab. Sterile filter paper discs (5 mm diameter) were kept on the above plates at equidistance. Varying volumes (2, 5 and 10 μ L) of rhizome oil was loaded over the sterile filter paper discs. The plates were incubated at 37°C for 18-24 h for bacteria, 48 h for fungi and observed for a zone of clearance around the discs which indicated positive microbicidal activity of the oil. All the experiments were carried out in triplicate.

Determination of MIC (minimum inhibitory concentration): Minimum inhibitory concentration of oil was determined by the tube dilution method (Pattnaik *et al.*, 1997). The oil was diluted with NBT and PDBT (Nutrient broth and potato dextrose broth supplemented with 0.75%

of Tween-20) to give oil concentration of 5 to 100 µg mL⁻¹. Fifty microliter of (fresh culture) over night growth of the test organisms in NB and PB was inoculated into 1 mL of NBT and PDT containing various concentrations of the oil. The tubes were incubated at 37°C, for 18-24 h (48 h for fungi) and the lowest concentration inhibiting bacterial and fungal growth (no turbidity) was noted as MIC.

Determination of bactericidal and fungicidal effect: In order to evaluate the effect (microbicidal /microbiostatic) of the oils, one loop from the MIC tube was sub cultured on to the NA and PDA plates which were then incubated at 37°C over night to check whether the oil merely had bactericidal or fungicidal activity i.e., no growth on subculturing.

Determination of MKT (Minimum killing time): This experiment was designed to determine the time required to kill the bacteria *in vitro* by the oil. One milliliter of NB, supplemented with 0.75% of DMSO and 1 mL of PDB supplemented with 0.75% of DMSO at MIC level of the oil was prepared and inoculated with 0.1 mL of freshly grown test organisms and incubated at 37°C. One loop of the sample from the above test tubes were sub cultured onto NA plates at 0, 5, 10, 15, 30, 45, 60, 90, 120, 180 min intervals and incubated overnight. Two sets of tubes were incubated for each test organisms from which subculturing were carried out alternatively (to avoid time lapse during subculture).

Statistical analysis: Each experiment was carried out in triplicate. The data were statistically analyzed using software SPSS 10.0. A least significant difference (LSD 0.05) was used to test the effects of essential oil through a general linear model. The test was statistically significant at p<0.05.

RESULTS AND DISCUSSION

In vitro antimicrobial property test of essential oil: The yield of essential oil from the rhizome of Roma cultivar was found to be 4%. From the preliminary screening studies by disc diffusion method, it was observed that the test pathogens were susceptible to the oil. However, differences in the zone sizes were observed with different pathogens (Table 1). Oil showed highest activity against *S. aureus* (IZD 49.83±0.76 mm), followed by, *C. albicans* (IZD 30±1), *A. niger* (IZD 25.33±0.57) and least activity against *P. aeruginosa* (IZD 14±1). The oil was significantly effective (p<0.05) against the test pathogens at all volumes. As evident from the Table 1, the IZD value for standard antibiotic used was invariably less than the corresponding IZD value obtained for essential oil against all pathogens except *P. aeruginosa*. Bacterial and fungal susceptibility towards the oil was observed at 2.5 µL per disc but higher concentrations 10 µL showed larger zones of

Table 1: Antimicrobial activity of roma rhizome oil by Disc Diffusion Method (DDM)

Microorganism	Mean zone sizes in mm by DDM* using different volumes (µL)			Inhibition zone diameter of gentamycin in mm	F-value
	2.5	5	10		
<i>S. aureus</i>	28±1	35.33±0.57	49.83±0.76	17±1	789
<i>P. aeruginosa</i>	8±1	12.33±0.57	14±1	25.67±0.57	256.3
<i>C. albicans</i>	14.67±0.57	24.67±0.57	30±1	27.67±0.57	273.5
<i>A. niger</i>	10.33±0.57	16.33±0.57	25.33±0.57	25±1	316.2

*Mean±standard deviation (SD) where n = 3 and data is significant at p<0.05

Table 2: MIC values for oil against tested organisms

Microorganism	MIC of oil ($\mu\text{L mL}^{-1}$)
<i>Staphylococcus aureus</i>	1.95
<i>Pseudomonas aerogenosa</i>	7.81
<i>Candida albicans</i>	5.50
<i>Aspergillus niger</i>	6.70

inhibition (Table 1), when tested by agar plate technique against all four microbial stains. The MIC of the oil against different test organisms was found to vary from $1.95 \mu\text{L mL}^{-1}$ against *S. aureus* to $7.81 \mu\text{L mL}^{-1}$ against *P. aerogenosa* amongst the pathogens analysed (Table 2). Though a variation was observed in the diameter of zones of inhibition at different concentration of oil and the MIC values for all the test pathogens but MKT result shows that *S. aureus* was killed soon after 15 min which indicates its microbicidal effect whereas rest other species killed after 12 h.

Experimental verification for validation of ethnological use of medicinal plants *in vitro* and *in vivo* has been one of the principal criteria of drug discovery worldwide since centuries. Validation reports like the attempt taken in work in support of ethological use of turmeric against pathogens responsible for eye infections across the globe including India, having a rich history of ethno-medicinal use of turmeric, are few. Ethno medicinal use of turmeric rhizome (popularly known as Ajo, Laali pupa or Obedo) by Yorubas of North Central Nigeria for human ailments is reported but not is supported with any information corroborated with experimental verification. (Maurice, 1993; Gul *et al.*, 2004) tried to experimentally validate the use of turmeric in Unani treatment in Pakistan against UTI infecting pathogens using resins and other extracts but found no activity to support essential oil against pathogens responsible for eye infections. Bacterial and fungal susceptibility towards the oil was observed at $2.5 \mu\text{L}$ per disc but higher concentrations at $10 \mu\text{L}$ per disc showed larger zones of inhibition when tested by agar plate technique which was found to be larger than activity shown against the antibiotic gentamycin against all the pathogens except *P. aeruginosa* (Table 1) which is found to be little more susceptible as compared to other three microbial strains. Only report on antibacterial activity of turmeric rhizome oil exhibits lower activity against *S. aureus* and *P. aeruginosa* but lacks the chemical constituent analysis of oil (Singh *et al.*, 2002). Such lower in antimicrobial activity might be due to rhizome oil used from a cultivar not having good quality in oil constituent in comparison to that of roma cultivar with turmerone as major constituents, as it has been established that the constituent of essential oil has got major role in attributing to its biological activity (Singh *et al.*, 2005). Chattopadhyay *et al.* (2004) have reported antimicrobial activity against *S. aureus* which is in agreement with our work but restricted to only one bacteria and the report also excludes MKT and microbicidal effects of essential oil against microbes causing eye infections. Curcuma oil showed positive activity against bacteria *S. aureus* including two more species which are not eye infecting pathogen (Chopra *et al.*, 1941). The results of this study suggest that the antimicrobial activity of the rhizome essential oil of roma cultivar is bactericidal against *S. aureus*. The oil was significantly effective ($p < 0.05$) against the test pathogens (Table 1). Antimicrobial activity of essential oil of turmeric with high tumerone content was reported by Singh *et al.* (2005) but the strains used were other then infecting eye except one *A. niger*. Babu *et al.* (2007) and Amphawan *et al.* (1995) have published separately their work denoting the antifungal activity of turmeric rhizome oil against species responsible for causing infections other than eye.

The results were highly significant for all the treatments, determining MICs, MKTs and even when the activities compared with standard antibiotics. In general, there seemed to be overall

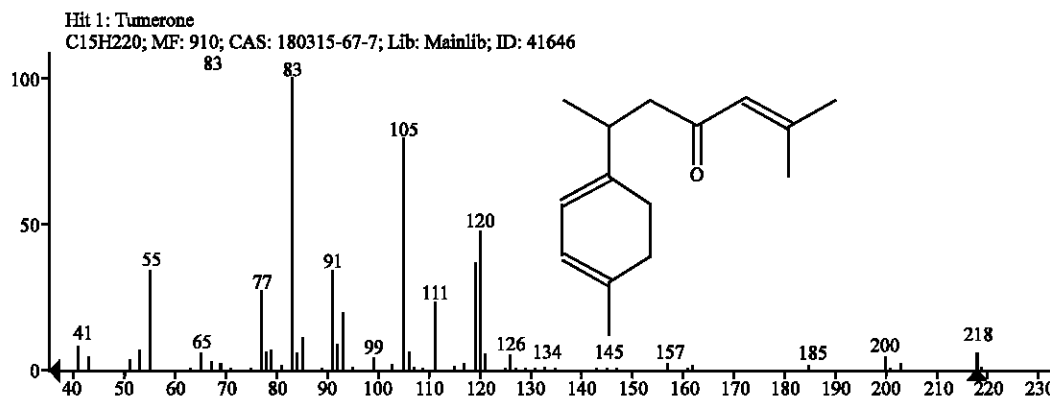


Fig. 1: Mass spectrum and structure of Ar-turmerone

agreement between the size of inhibition zones obtained by the Disc Diffusion Method (DDM) and the Minimum Inhibitory Concentration (MIC) values, i.e., larger zones of inhibition correlated with lower MIC values. This relationship between inhibition zones and MIC values has been reported in literature while studying the antibacterial activity of essential oils (Rath *et al.*, 2005). Roma rhizome essential oil was found contain 49.76% of turmerone (Fig. 1) which is supported by the report showing presence of ar-turmerone as most abundant constituent in rhizome essential oil of South-West Nigerian grown turmeric by Ajaiyeoba *et al.* (2008), Usman *et al.* (2009). Ar-turmerone has been reported as the major constituent of rhizome oil of turmeric of different origin (Singh *et al.*, 2005) but all these reports are not supported with any antimicrobial activity study against eye infecting pathogens.

Better activity of roma rhizome oil could be due to presence of high percentage of ar-turmerone (49.76%). Some reports support our inference that turmerone is responsible for showing antimicrobial activity against specific pathogens (Amphawan *et al.*, 1995). Previous studies have already shown the growth inhibition activity of *Curcuma longa* rhizome essential oil on different microorganisms.

However, this is the first time that the bactericidal activities of *Curcuma longa* rhizome essential oil have been demonstrated against eye disease causing pathogens. The results appear promising, for possible use of this rhizome oil of roma cultivar as bactericidal agents (Baratta *et al.*, 1998), more particularly eye infections which are very sensitive organs. The use of antibiotics causes severe side effects like hypersensitivity reactions, gastric disturbances, ototoxicity and nephrotoxicity (Cosgrove *et al.*, 2009). Different antibiotics such as chlorotetracyclin, oxytetracycline and chloramphenicol at low concentrations have been used to prevent these infections. These antibiotics have been proved to have severe side effects, now-a-days consumers are concerned about the ill effects due to use of synthetic antibiotics. When safety of synthetic products is questioned, natural compounds of plant origin may appeal to the public.

CONCLUSION

Probably in our investigation, for the first time we have documented the antibacterial activity of rhizome oil of roma cultivar against eye infection causing pathogens, experimentally supported with low concentration of oil needed to affect the growth of a range of microorganisms responsible for dreaded eye diseases within a short period of killing time supplemented with information on its

major chemical constituent, α -turmerone. Furthermore, essential oil of rhizome of roma cultivar proved to have bactericidal properties at low concentrations and most of the essential oil components possess antioxidant properties holds a promise as an alternate to expensive, harmful antibiotics against these pathogens. Of course, other studies are highly necessary to study the toxicity of these oils in order to set an appropriate formulation like eye drop for this purpose.

ACKNOWLEDGMENT

The authors are grateful to Prof. Dr. S.C. Si, Dean, Centre of Biotechnology and Prof. Dr. M.R. Nayak, President, Siksha O Anusandhan University for providing financial support and encouraging throughout.

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