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Comparative evaluation of anti-oxidant activity of ethanolic and aqueous extracts of different medicinal plants to be used as therapeutics in aquaculture

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Abstract
The present study was conducted to elucidate the anti-oxidant effect of different herbal extract and the imidazole antifungal drug miconazole nitrate (MCZ). For the experiment, different herbs with medicinal properties were collected. Extracts were prepared from herbs and their antioxidant activity was evaluated using 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) and Alkaline dimethyl sulfoxide (DMSO) radical scavenging assay. To find out the best extract of herbal plant by determining the antioxidant activity or their ability to scavenge free radicals, diverse varieties of herbal plants with medicinal properties were collected like *Curcuma longa* (turmeric) rhizome, *Azadirachta indica* (neem) leaves, *Allium sativum* (garlic) cloves, *Zingiber officinale* (ginger) rhizome. Herbal extract was prepared from the herbs obtained using ethanol and water as solvent. Ethanolic and aqueous extractions of all the selected plant parts were done and the yield percentages of the extracts were calculated. The highest yield obtained was of ethanolic garlic cloves extract (15.89%) and lowest being the aqueous neem leaves extract (6.34%). Among the different herbal extract, ethanolic turmeric extract was found to be more active and shows significantly higher activity compared to others, both in DPPH and alkaline DMSO scavenging assay whereas in ethanolic garlic extract has lower activity was observed.

Keywords: Herbs extract, antioxidant, herbal plants, medicinal properties

1. Introduction
Aquaculture is an immensely rising sector and its products possess an incredibly valuable source of animal protein and essential nutrients. Over the last 30 years the aquaculture sector has shown a rapid increase to meet the increase in world populations and human needs. Fish and other aquatic products supply at least 20% of protein intake in developing countries (Bene *et al*., 2007) [3] and more than 50% of the protein and minerals intake is in the poorest countries of Africa and South Asia (Richardson *et al*., 2011) [21]. Presently, India ranks second in the world in total fish production with an annual fish production of about 12.60 million metric tons during 2017-18, of which nearly 65% is from inland sector and about 50% of the total production is from culture fisheries, constituting 6.3% of the global fish production (FAO, 2019) [11]. Though the aquaculture production has increased substantially with the successful implementation of different intensified polyculture practices at a large scale, the industry has faced a considerable set back, because of loss due to sudden disease outbreaks. These outbreaks not only include trans-boundary aquatic animal diseases but also many infections caused by viruses, bacteria, fungi, parasites and other unidentified emerging pathogens. Disease thus became a primary concern in the culture of many aquatic species impeding both economic and social development. The loss due to some of these old and new infective diseases is so significant that it has almost paralyzed the growth of aquaculture industry and prompted many countries to assess the estimates of disease loss. This loss was mostly due to poor understanding of the farmers on disease diagnosis and health management. Considering the potential harm of veterinary drug treatments on the environment and human health and in some cases their limited efficacy, disease management should concentrate on harmless, preventive and lasting methods. Medicinal plants are promising to be an important source of therapeutics in fish culture since these products provides a cheaper source for treatment, eco-friendly and greater accuracy without causing toxicity. Plants are rich in a wide variety of secondary metabolites of phytochemical constituents such as tannins, terpenoids, alkaloids and flavonoids, which act against different diseases (Ravikumar *et al*., 2010) [20]. Though much work has been done on ethno medicinal plants, there is still need to seek plants with medicinal

value to combat diseases. Although active ingredients may occur in lower concentrations, plant extracts may be a better source of antimicrobial than synthetic drugs. Therefore to overcome the problem this experiment was conducted to find out the best extract of herbal plant by determining the antioxidant activity or their ability to scavenge free radicals.

2. Material and Methods

2.1 Collection and Preparation of extract

The experiment was conducted to find out the best extract of herbal plant by determining the antioxidant activity or their ability to scavenge free radicals. Diverse varieties of herbal plants were collected like *Curcuma longa* (turmeric) rhizome, *Azadirachta indica* (neem) leaves, *Allium sativum* (garlic) cloves, *Zingiber officinale* (ginger) rhizome. Herbal extract was prepared from the herbs obtained using ethanol and water as solvent.

Extract was prepared from *Curcuma longa* (turmeric) rhizome, *Azadirachta indica* (neem) leaves, *Allium sativum* (garlic) cloves, *Zingiber officinale* (ginger) rhizome following Singh *et al*. (2003) [25]. First the selected herbs parts were washed with clean water, dried and chopped into smaller pieces, this was further dried in shade till almost all the moisture were removed. The dried samples were then grinded into fine pieces and soaked in water and alcohol at the ratio of 1:10. The mixtures were filtered using Whatman filterpaper (No.1) after constant mixing in rotary shaker for 48 hours and centrifuged at 5000 RPM for 10 minutes. The supernatant was collected and kept in an amber bottle at 4 ºC until further use. The solvent was then evaporated in rotary evaporator until it become semi solid and finally dried in freeze drier for 24 h. The extracts were then kept in -20 °C till further use. The yield of the extract was calculated as follows:

$$\text{Yield (\%)} = \frac{\text{Weight of extract}}{\text{Weight of dry sample}} \times 100$$

2.2 Making different extracts of herbal nutraceutical

The different extracts were made to evaluate the antioxidant activity *in vitro* Table 1.

Table 1: Different herbal extracts for *in vitro* analysis

Sl. No.	Herbal extract
1.	Aqueous turmeric (AT) extract
2.	Ethanolic turmeric (ET) extract
3.	Aqueous neem (AN) extract
4.	Ethanolic neem (EN) extract
5.	Aqueous garlic (AG) extract
6.	Ethanolic garlic (EG) extract
7.	Aqueous ginger (AZ) extract
8.	Ethanolic ginger (EZ) extract

2.3 Property estimation of different herbal nutraceutical

The antioxidant activity of different herbal extracts was assessed using DPPH (1, 1-Diphenyl-2-picrylhydrazyl) and alkaline DMSO radical scavenging method.

DPPH radical-scavenging activity was performed by the method described by Akter *et al*. (2010) [1]. For each determination, the stock solution (1mg ml^{-1}) was diluted to a dilution (200 µg ml^{-1}) with absolute (v/v) ethanol. An aliquot of each dilution (0.5 ml) was mixed with ethanolic solution of DPPH (5 ml, 0.06 mM). The mixtures were shaken vigorously and incubated at 37 °C in the dark for 30 min. At the same time, a control containing absolute (v/v) ethanol (0.5 ml) and ethanolic solution of DPPH (5 ml, 0.06 mM) was run. The absorbance was measured at 517 nm against ethanol as a blank. The percentage of DPPH scavenging was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Abs. control} - \text{Abs. sample}}{\text{Abs. control}} \times 100$$

The reduction of Nitro Blue Tetrazolium (NBT) by superoxide was determined according to the method of Ruch *et al*. (1989) [22]. To the reaction mixture containing 1 ml of alkaline DMSO, 0.3 ml of the samples at various concentrations and 0.1 ml of NBT (0.1 mg) were added. The reaction mixture was incubated for 10 min at room temperature. After incubation, absorbance was read at 560 nm. The percentage scavenging activity of alkaline DMSO was calculated using the following equation:

$$\text{\% radical scavenging activity} = \frac{\text{Abs. control} - \text{Abs. sample}}{\text{Abs. control}} \times 100$$

3. Results

The yield percentage of aqueous and ethanolic extract of *Curcuma longa* (turmeric) rhizome, dried leaves of *Azadirachta indica* (neem), rhizome of *Zingiber officinale (ginger)* and cloves of *Allium sativum* (garlic) are given below in Table 2.

Table 2: Total yield of the different herbal extracts in ethanol and aqueous solvents, expressed in the term of percentage (%)

S. No.	Type of herbal extract	Yield (%)
1.	Aqueous turmeric (AT) extract	7.81
2.	Ethanolic turmeric (ET) extract	12.31
3.	Aqueous neem (AN) extract	6.34
4.	Ethanolic neem (EN) extract	9.11
5.	Aqueous garlic (AG) extract	13.08
6.	Ethanolic garlic (EG) extract	15.89
7.	Aqueous ginger (AZ) extract	8.99
8.	Ethanolic ginger (EZ) extract	9.54

3.1 DPPH (1, 1-Diphenyl-2-picrylhydrazyl) activity

DPPH radical scavenging activity of individual herbal extract ranges from 16.25±1.64^{e} % in ethanolic garlic cloves extract to 56.14±1.45^{a}% in ethanolic turmeric extract. DPPH radical scavenging activity (%) for different types of herbal extracts are presented in Table 3 and Fig. 1.

3.2 Alkaline DMSO radical scavenging activity

Percentage radical scavenging activity assayed by alkaline DMSO of individual herbal extracts ranges from 67.56±1.31% in aqueous neem to 84.54±1.13% in aqueous ginger rhizome extract. Alkaline DMSO radical scavenging activities (%) for different types of herbal extracts are presented in Table 3 and Fig. 2.

Table 3: Percentage radical scavenging activity of different herbal extract

S. No.	Different plant extracts and drug	% Inhibition (200 µg ml^{-1})	
		DPPH	Alkaline DMSO
1.	MCZ	12.65±0.51^{a}	07.29±2.65^{a}
2.	AT	27.91±1.56^{b}	73.25±1.85^{b}
3.	ET	56.14±1.45^{bc}	79.41±0.92^{b}
4.	AN	36.46±3.08^{b}	67.56±1.31^{b}
5.	EN	29.02±1.15^{b}	73.21±0.83^{b}
6.	AG	20.13±3.27^{a}	77.92±0.91^{b}
7.	EG	16.25±1.64^{a}	81.39±0.82^{b}
8.	AZ	23.14±3.79^{a}	84.54±1.13^{b}
9.	EZ	32.52±2.55^{a}	80.31±1.45^{b}

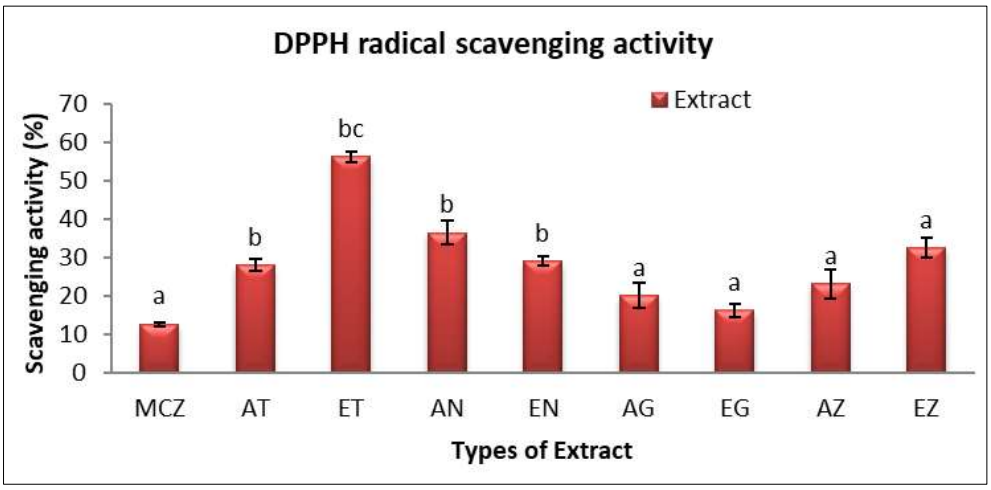

Fig. 1 DPPH radical scavenging activity (%) of different types of herbal extract and antifungal drug MCZ. a, b indicates statistically significant difference *(p*<0.05*)* when compared with control

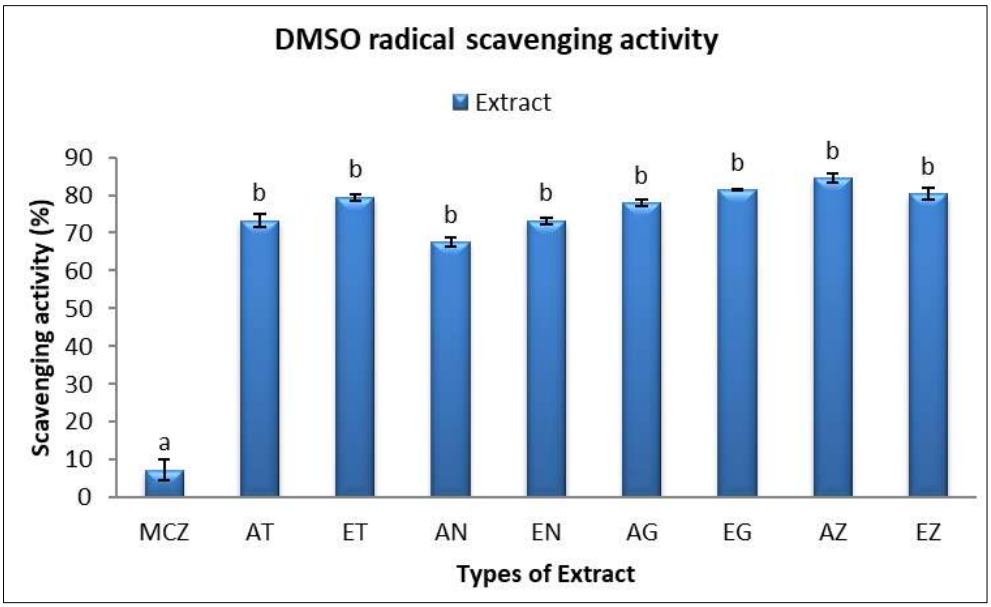

Fig 2: Alkaline DMSO radical scavenging activity (%) of different types of herbal extract and antifungal drug MCZ. a, b, c indicates statistically significant difference (*p*<0.05) when compared with the control

4. Discussion

After considering the harmful effect of unregulated usage of veterinary drugs in aquaculture either on aquatic animals or on the environment and human health, herbal plants with medicinal properties came as a promising and alternative method for the control of fish disease. These medicinal plants have not only potential to provide treatment to infected fish as chemotherapeutics but can also be used as feed additives (Wang *et al.*, 2015) [30], possessing a wide variety of nutrients and chemical compounds (Chang, 2000) [6]. These plants when administered either directly or in extract form have been proven as growth promoter, appetite stimulator, immuno-stimulant, antimicrobial, antifungal and anti-stress in fish (Chitmanat *et al.*, 2005; Citarasu, 2010; Chakraborty and Hancz, 2011) [7,8 and 5]. Natural product chemistry has developed globally in the attempt to discover even more

efficient and cost-effective drugs with lower side effects. Despite the milder effects of many natural products on the fish and the environment, they may have longer lasting effects than synthetic drugs (Awaad, 2009) [2]. When medicinal plants are used as a treatment in combination with the synthetic drugs, they can act as synergic medicine, in which active compounds interact simultaneously and their action can complement or damage others or neutralize their possible negative effects.

In this experimental set up, the *in vitro* screening of the different varieties and parts of herbs were done by analysing their antioxidant activity or their ability to scavenge free radicals with the imidazole drug MCZ. Rhizomal parts of *Curcuma longa* (turmeric), *Azadirachta indica* (neem) leaves, *Allium sativum* (garlic) cloves, *Zingiber officinale* (ginger) rhizome were identified and collected from the local market for the preparation of their extracts using aqueous and ethanolic solvents. In this experiment, firstly the antioxidant potency of different herbal extracts was determined to know their ability to scavenge free radicals which causes an oxidative stress. The free radicals generally known as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Halliwell and Gutteridge, 2015) [14] play an important role in biological system they can either be beneficial or harmful for the system (Valko *et al*., 2007) [29]. At low or moderate levels, ROS and RNS exert beneficial effects on cellular responses and immune function. At high concentrations, they generate oxidative stress, a deleterious process that can damage all cell structures (Young and Woodside, 2001; Halliwell and Gutteridge, 2015) [31 and 14]. Oxidative stress can be neutralized by antioxidant generated naturally inside the body or externally supplied through foods. Antioxidants from diet play an important role in helping endogenous antioxidants for the neutralization of oxidative stress (Pham-Huy *et al*., 2008) [19]. So, different herbal extracts were prepared which were known to possess antioxidant property based on a study conducted by several researchers like *Azadirachta indica* leaves (Kiranmai *et al*., 2011; Pandey *et al*., 2014) [15 and 16], *Allium sativum* cloves (Boonpeng *et al*., 2014; Wang *et al*., 2015) [19 and 20], *Zingiber officinale* rhizome (Danwilai *et al*., 2017; Stoilova *et al*., 2007) [4 and 28] and *Curcuma longa* rhizome (Singh *et al*., 2010; Park *et al*., 2019) [24 and 17]. Ethanolic and aqueous extractions of all the selected plant parts were done and the yield percentages of the extracts were calculated. The highest yield obtained was of ethanolic garlic cloves extract (15.89%) and lowest being the aqueous neem leaves extract (6.34%). The antioxidant and free radical scavenging activity of the herbal extracts was investigated against *in vitro* models. Since at least two methods should be employed in order to evaluate the total antioxidant activity (Gulcin *et al.*, 2005) [13] we had carried out DPPH and alkaline DMSO radical scavenging assay.

The DPPH is a nitrogen stable free radical, which has been widely accepted tool for estimating free radical scavenging activities of antioxidants in a relatively short time compared with other methods (Sancehz-Moreno, 2002) [23]. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soare *et al*., 1997) [26]. Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive species (Halliwell and Gutteridge, 2015) [14]. The superoxide radical is known to be produced *in vivo* and can result in the formation of hydrogen peroxide via dismutation reaction. Moreover, the conversion of superoxide and hydrogen peroxide into more reactive species. The superoxide radical scavenging activity was examined by alkaline DMSO assay. Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive species (Halliwell and Gutteridge, 2015) [14]. The superoxide radical is known to be produced *in vivo* and can result in the formation of hydrogen peroxide *via* dismutation reaction.

Based on the *in vitro* antioxidant study using DPPH and alkaline DMSO all the selected extract is found to be an efficient scavenger of superoxide radical. Among the different herbal extract, ethanolic turmeric extract was found to be more active and shows significantly higher activity compared to others, both in DPPH and alkaline DMSO scavenging assay whereas in ethanolic garlic extract has lower activity was observed. When compounds are used in the combinations, the antioxidants act in a regenerating manner, with either the stronger regenerating the weaker, i.e., antagonistic effect or the weaker regenerates the stronger, synergistic effect (Peyrat-Maillard *et al*., 2003) [18]. The reaction rates of antioxidants, the polarity of the interacting molecules and the effective concentration of the antioxidants at the site of oxidation are also some of the other factors effecting the interaction of compounds in combination (Frankel *et al*., 1994; Cuvelier *et al*., 2000) [12 and 9]. The interactions among different antioxidant components can be synergistic, additive or antagonistic (Sonam and Guleria, 2017) [27].

5. Conclusion

It is crystal clear that the use of herbal plant extract may be successful to use antioxidant combinations in which the antioxidants produce a synergistic effect. The results defined that ethanolic turmeric extract *Curcuma longa* is a syllable important plant for more experiments in fish microbiology. Although the antioxidant activity of different herbal extracts were observed further research will be needed to know the active compound responsible for scavenging radicals, their interaction and mechanism of action with the anti microbial components *in vitro* and *in vivo*.

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