ANTIOXIDANT AND CYTOTOXIC PROPERTIES OF BLACK TURMERIC (*Curcuma Caesia* Roxb.) RHIZOME EXTRACT

A dissertation submitted for the partial fulfillment of Master's Degree in Botany

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DECLARATION

I hereby declare with immense pleasure and satisfaction that this dissertation work entitled **"Antioxidant and Cytotoxic properties of Black Turmeric (*Curcuma caesia* Roxb.) rhizome extract"** was carried out by me under the supervision of Assoc. Prof. Dr. **Krishna Pant**, Central Department of Botany, Tribhuvan University, Nepal.

……

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RECOMMENDATION

This is to certify that the dissertation entitled **"Antioxidant and Cytotoxic properties of Black Turmeric (*Curcuma caesia* Roxb.) rhizome extract"** is the bonafide work of Mina K.C carried out under my guidance, in the partial fulfillment of the requirement of Master of Science in Botany with "Plant Biotechnology and Genetic Engineering" as major. This work is primarily based on the result of her research work and has not been submitted to any other institutions for the award of any other degree. I recommend the dissertation work to be accepted for the partial fulfillment of Master of Science in Botany (M.Sc. Botany).

…….....

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LETTER OF APPROVAL

The M.Sc. dissertation entitled **"Antioxidant and Cytotoxic properties of Black Turmeric (*Curcuma caesia* Roxb.) rhizome extract"** submitted at Central Department of Botany, Tribhuvan University by Mrs. Mina K.C. has been accepted for the partial fulfillment of Masters of Science in Botany.

EXAMINATION COMMITTEE

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ABBREVIATIONS AND ACRONYMS

DCM - Dichloromethane

DE - Dried extract

DECC - DCM extract of *Curcuma caesia*

DMSO - Dimethyl sulfoxide

DPPH - 2, 2- Diphenyl-1-picrylhydrazyl

DPY - Dry powered yield

E / EYD - Extract/ Dried extract yield

FW - Fresh weight

g - grammae

HECC - Hexane extract of *Curcuma caesia*

IC_{50} - Half Inhibition Concentration

LC_{50} - Half Lethal Concentration

MEFCC - Methanol extract fraction of *Curcuma caesia*

mm - millimeter

mg - miligrammae

µg - microgrammae

ml - milliter

mM - millimolar

NaCl - Sodium Chloride

nm - Nanometer

ROS - Reactive oxygen species

UV – Ultravoilet

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ABSTRACT

The rhizomes of *Curcuma caesia* have got wide range of attention on various antipyretic, analgesic, antimutagenic, neuropharmacological, antibacterial and antifungal pharmacological activities. It is well known for its peculiar characteristics other than yellow turmeric. With the increasing trend on natural products in modern phytomedicines, the antioxidant and cytotoxic activities of *Curcuma caesia* Roxb. rhizome fractions were evaluated. The DCM fraction showed highest activity with DPPH assay (78.17%) comparable to the ascorbic acid (88.01%) at 100µg/ml concentration with IC_{50} 42.72±1.06 µg/ml for DCM fraction followed by MEFCC (109.25±3.4 µg/ml) and HEFCC (214.87±0.78 µg/ml) respectively. DCM fraction exhibited more potent antioxidant, subjected to purify active compound with Sephadex LH-20 column chromatography and thin layer Chromatography (TLC), revealed different phytochemical compound with different R_f values ranging from 0.21 to 0.56. Moreover, based on antioxidant activity, the rhizome fractions were subjected to their cytotoxic properties against brine shrimp napaulii. The highest degree of lethality was observed in Methanol fraction (LC_{50} 11.42 µg/ml), while low at DCM fraction (LC_{50} 37.12 µg/ml). Lower the LC_{50} value of extract, higher its cytotoxic effect. As all the extract fractions in both the assay were found to be react in dose dependent manner. The results thus obtained in the present study indicated that *Curcuma caesia* Roxb. rhizome is a rich source of phytochemicals. This could justify the use of plant species in traditional medicines for the treatment of various ailments.

CHAPTER ONE: INTRODUCTION

1.1. Background

1.1.1. Antioxidants and antioxidant activity

An antioxidant can be broadly defined as the molecules that inhibit or delay the oxidative damage in cellular level of living organisms. And such inhibiting activities against oxidative damage are known as antioxidant activity. Oxidative damage is an important factor in pathogenesis of various chronic diseases and it results from the disparity between the generation of free radicals and the antioxidant ability of the organisms (Chiavaroli *et al*., 2011). Antioxidants are substances able to counteract or reduce molecular and cellular reactive oxygen species (ROS) injury.

Oxidation is the chemical process that transfers electrons from one molecule to an oxidizing agent. Oxidation reaction is known to produce free radicals in which they are highly reactive species and contain one or more unpaired electrons in their outermost shell. The oxidation of lipid, protein and carbohydrates by toxic reactive species, cause DNA mutation and lead to damage the cell/tissue and death (Desmarchelier and Lisbod, 1999). Antioxidants thus react with these free radicals and terminate the reaction thereby removing free radicals intermediate, inhibit other oxidations being oxidizing themselves. Cellular system has developed many endogenous antioxidants such as superoxide dismutase (SOD), catalase, glutathione, glutathione peroxidases and reductase, and non-enzymatic antioxidants like vitamin E (tocopherols and tocotrienols), vitamin C etc. to neutralize the free radicals (Ahmad *et al*., 2010). This has triggered to search for effective antioxidant agents from various sources including plants.

Since long plants have been a source of food and medicines, either in the form of conventional preparations or as pure active principles. Most of the observed therapeutic effects of plants have been associated to their potent antioxidant activity. They produce diverse range of phytochemicals which are grouped as terpenoids, alkaloids, glycosides, steroids, carotenoids and phenolics of biosynthetic origin. Among these, phenolics are often known as polyphenols that possess marked antioxidant activity

(Carocho and Ferreira, 2013). Major form of plants phenolics are hydroxycinnamic acid, flavonoids, lignans, stilbenes and tannins (Myburgh 2014; Duthie and Kyle, 2000). It has commonly been assumed that the antioxidant capacity of phenolics will increase with the number of free hydroxyls and conjugation of side chains to the aromatic rings (Morgan, 1997).

It is to be believed that two-thirds of the world's plant species have medicinal importance, and almost all have an excellent antioxidant potential (Li *et al*., 2008; Awah *et al*., 2013; Krishnaiah *et al*., 2011). The quest in the exogenous plant antioxidants was first brought to mind by the discovery and consequent isolation of ascorbic acid from plants (kasote *et al*., 2015). Since then, they have shown promising antioxidant activity in both invivo and invitro investigation. Natural antioxidants are proven to have multiple biological significant activities in anti-inflamatory, anti-bacterial, anti-allergic, antiviral, antitumor, anticancer assay (Li *et al*., 2008; Velioglu *et al*., 1998).

1.1.2. Antioxidants vs pro-oxidants

Antioxidants compound with their antioxidant activity carried out with large number of different assays, all of them with some pros and cons. Singh *et al*. (2011) said that antioxidant gone through miracle to marvelous molecules and finally to physiological ones. Without any doubt, these molecules have better role in metabolism and cell protection but conflicts on the role of antioxidant and pro-oxidants strikes on researcher mind to go through depth. An excess of any one type of antioxidant in the absence of balance with the others may actually be counter-protective. It was explained that most of the dietary antioxidant acts as pro-oxidants and all it depends on the concentration and nature of surrounding molecules (Villanueva and Kross, 2012). Alpha-tocopherol being powerful antioxidant agents shows pro-oxidant at high concentration (Carocho *et al*., 2013).

Moreover, the antioxidants do have an impact on health, but the debate arose in way of administration (food vs supplement) and quantity. The priority of antioxidants varied with diseases condition because the types of reactive oxygen species (ROS) generated are differ and also diverse levels of specific antioxidants exist within the different tissues of the body (Bland, 1995). So, antioxidants display a useful role in human

homeostasis, but acts as pro-oxidants when these are not present at right place at the right concentration at the right time (Pal *et al*., 2014).

1.2. Relationship between antioxidant and cytotoxicity

Over the time, interest on broad spectrum of cytotoxicity and cell viability assay is currently used in the field of pharmacology and toxicology. Medicinal plants being important natural sources of therapeutic agents are well known for their less toxic effects than synthetic compounds. In the same way, interest in toxic plants is increasing because these plants contain bioactive compound of medicinal virtues and their bioactivity to discover the most potent ones for the desired effects. In this regard, many former researches reported that cytotoxic effect of medicinal plants are somehow associated or attributed by their antioxidant activities.

Moreover plants been exploited for the anticancer treatment in tumor biology since long time. It is not always the case that toxic bioactive compound be useless in pharmacological fields, rather they may be high demanding to treat complicated ailments in medical fields. Though, side effects limit the use of drugs in medicinal fields but it is undeniable that drugs without side effect are not effective. Sometimes side effect has got better effect on carcinoma cell. So it is quite encouraging that potential antioxidant activity directs its cytotoxic effects and potent antitumor activity. Viable cancer causing cells have been recovered to normal by gradual decrease in oxidative stress due to cytotoxicity of antioxidant compound (Karmakar *et al*., 2013).

1.3. Determination of antioxidant activity and cytotoxic activity

Plants extracts have revealed a number of secondary metabolites with antioxidant activity. There has been an increased interest in identifying such compound from plants with low or no side effects for use in medicines, preventives and food industry. These protective and medicinal effects of antioxidants have received increasing attention within biological, medical and nutritional fields, resulting in the requirements of simple, convenient and reliable antioxidant capacity determination methods. Therefore, antioxidant activity by DPPH free radical scavenging assay considered to be rapid and effective methods (Cheng *et al*., 2002; Koleva *et al*., 2002). However, a broad spectrum of cytotoxicity assay currently used in the field of phytochemical extraction and analysis of plant derived phytochemical compound. The Brine shrimp bioassay is

considered to be preliminary, quick, in-house and inexpensive method for screening and fractionation monitoring of our physiologically active plant extract based on the ability to kill laboratory cultured brine shrimp (*Artemia salina*) (Meyer *et al*., 1982).

Antioxidant assay by DPPH (2, 2-Diphenyl-1-picrylhydrazyl) is a purple coloured stable nitrogen radical which exhibits its highest absorbance in solution of methanol or ethanol at 515 nm to 520 nm. When the plant extract containing antioxidant compound allowed to react alcoholic $DPPH^{\bullet}$ (DPPH radical in ethanol or methanol solution) then yellow coloured 2,2- diphenyl-1-picryl hydrazine or non-radical form DPPH-H is formed as in the equation below and decrease in absorbance occurs. And this phenomenon of decrease in absorbance of alcoholic DPPH is applied to determine radical scavenging or antioxidant activity of specific compound or extract (Koleva *et al*., 2002).

$$DPPH^{\bullet} + AH \longrightarrow DPPH\text{-}H + A^{\bullet}$$

1.4. Study species and their medicinal properties

Curcuma caesia Roxb. (Local name: Kalo besar) a member of the family Zingiberaceae and commonly known as black turmeric. It is well known for its peculiar characteristics; bluish black rhizome with camphoraceous sweet odour and a deep farraginous purple color present in the middle region of lamina. This is the perennial rhizomatous herbaceous medicinal plant native to North-East India (Reenu *et al*., 2015) including the tropical and sub-tropical region of Nepal. In genus *Curcuma*, there are more than 80 species of rhizomatous herbs. Wild turmeric, turmeric, Barkhe sarro (*Curcuma augustifolia*) are some of the medicinal plants belonging to this genus *Curcuma*. In Nepal, black turmeric is mainly found in the wild, but its cultivation over the year is prominent due to their aesthetic, food and medicinal values. The profound medicinal importance of rhizome laid this species under the threat of vulnerable since natural habitat is being destroying through several human activities like bio piracy, overexploitation and industrialization.

Similar to other member of genus *Curcuma*, it has also many therapeutic properties and is especially used in digestive complaint, relief in abdominal gas, cough and cold (Khare, 2007; Mangaly and Sabu, 1990). The species is also recognized for healing properties like wound, fever, vomiting, toothache, sprains and bruises, bronchitis,

tumors, piles, leprosy, leucoderma, asthma, epilepsy, smooth muscle relaxant, hemorrhoids, cancer, menstrual disorder, anti-helminthic, aphrodisiac, inflammation and gonorrheal discharges in various scientific studies (Amalraj *et al*., 1989; Craker and Simon, 1989; Arulmozhi *et al*., 2006). Its use is also recommended for auspicious value that it keeps evil spirits away if grown nearby house. This species has variety of chemical constituents such as flavonoids, glycosides, phenols, polysterols, alkaloids, carbohydrates, oils, amino acids, terpenoids, saponins and tannins (Sarangthem and Haokip, 2010; Pandey *et al*., 2003 and Paliwal *et al*., 2012), all these chemical constituents indeed solely responsible for various pharmacological activities.

Figure1. *Curcuma caesia* plants with flower.

1.5. Hypothesis

The hypothesis of the present study is that the extract of black turmeric rhizome with three different solvent fractions possesses antioxidant activity (DPPH scavenging assay) and cytotoxicity against brine shrimp napaulii.

1.6. Objectives

A. General objective

To evaluate the antioxidant and cytotoxic activity from the extract of *Curcuma caesia* Roxb. rhizome fractions.

B. Specific objectives

- ✓ To evaluate antioxidant activity from different solvent extract of rhizome.
- ✓ To asses TLC guided antioxidant test.
- ✓ To perform cytotoxicity of different solvent fractions.

1.7. Rationale of the study

Black turmeric is one of the most valued plants in existence today. They are valuable to Nepalese due to their aesthetic, food and medicinal properties. Several studies have been done regarding their ethno medicinal uses, phytochemical studies, fewer on conservation status and fewer biological properties on newly emerging diseases. But, the knowledge and information of pharmacological and biological activities of Nepalese Black turmeric is still overdue. There is the study reported that *Curcuma caesia* has the possibility to become important in the economic aspects because of higher phenolic compound than *C amada* (Sahu and Saxena, 2013).This study can provide sufficient knowledge about phytochemical classes, pharmacognostic properties and biological activity of this plant. Further the antioxidant and cytotoxic activity of rhizome extract will provide further validation with high quality clinical evidence of their medicinal importance. Besides, study will open floor for further research on various biological activity and knowledge based strategies to encourage for the conservation of this species.

CHAPTER TWO: LITERATURE REVIEW

Although many studies have been performed on the bioactive constituents present, still much more are to be evaluated and exploited.

2.1. Traditional medicine of rhizomes

So far, ethno medicinal practices have been used for the remedies of various human ailments. The genus *Curcuma* as sophisticated traditional medicines used in wide range but *Curcuma caesia* is very less known and untouched drug in today's scientific world.

Ethnomedically, *Curcuma caesia* are used to cure leprosy, wounds, impotency, fertility, toothache, vomiting, allergies, leucoderma, asthma, piles, bronchitis, epileptic, menstrual disorder, anthelmintic, aphrodisiac, gonorrheal discharges (Amalraj *et al*., 1989; Ravindran *et al*., 2007; Israr *et al*., 2012). The paste is applied on bruises, contusions, and rheumatic arthritis pains, anti-diarrhoetic and to get relief from stomach ache. In eastern India, the paste of fresh rhizome used in case of snake and scorpion bite (Tag *et al*., 2007). In Assam, fresh rhizome juice mixed with mustard oil and is given to cattle in dysentery (Saikia and Borthakur, 2010)

2.2. Phytochemical constituents in *Curcuma caesia*.

Earlier research on Curcuma species have shown the numerous phytoconstituents present in it. Most commonly found phytoconstituents is Curcumin.

Pandey *et al*. (2003) analyzed the volatile oil components in rhizome of *Curcuma caesia* by GC-MS that resulted in the identification of 30 components, representing 97.48% of the oil, with camphor (28.3%), ar-tumerone (12.3%), (Z)-Ocimine (8.2%), 1-ar-curcumene(6.8%), 1, 8-cineole (5.3%), element (4.8%), borneol (4.4%), bornyl acetate (3.3%) and curcumene (2.82%) as the major constituents. Similarly, same kind of outcome have been reported which comprises linalool (20.41%) and d- camphor(18.88%) as major component followed by ocimine (15.66%), 1-ar curcumene (14.84%), Zingiberol (12.60%), 1,8-cineole (9.06%) and α-borneol (7.8%) respectively (Banergee and Nigam, 1974; Rastogi and Malhotra 1996).

Simply phytochemical screening of n-hexene, petroleum ether (60:80), benzene, chloroform, ethyle acetate, methanol and water extract of rhizome *Curcuma caesia* revealed the presence of alkaloids, phenol, phytosterol, terpenoids, carbohydrates, glycosides, saponins, quinones, aminoacids and flavonoids (Donipat and Sreeramulu, 2015; Sarangthem and Haokip, 2010; Mukunthan *et al*., 2017; Paliwal *et al*., 2011)

Pakkirisamy *et al*. (2017) evaluated bioactive compound of *Curcuma caesia* Roxb using GC-MS and FTIR. As a result, α-Santalol (46.90%), Retinal (10.72%), Ar-tumerone(10.38%), Alloaromadendrene (5.93%), Megastigma-3,7(E),9-triene (4.80%), Benzene, 1-(1,5-dimethyl4-hexenyl)-4-methyl(4.38%), 5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)-(4.26%) Tricyclo [8.6.0.0(2, 9)] hexadeca-3,15-diene, trans-2,9-anti-9,10-trans-1,10 (3.26%) were found as major compound. While, N-H, O-H, C=C, C-H, C-O and CH3 functional group were identified after FTIR report.

2.3. Pharmacological activities of *Curcuma caesia* **Roxb.**

Former study regarding essential oil of *Curcuma caesia* reported the species having both antibacterial and antifungal activities (Rahman and Yusuf, 1996). Similary, the antifungal assay against *C. albican* was performed with the crude protein extract of *Curcuma caesia*. And, the better result showed with minimum inhibitory concentration observed to be 55µg/ml (Mannangatti and Narayanasamy, 2008).

Arulmozhi *et al*. (2006) evaluated anti-asthamatic property of *C.caesia.* The hydraulic extract of *Curcuma caesia* was tested for its relaxant effect in Guenia pig trachea and also in the presence of various receptor antagonists and enzyme inhibitors. The possible role of hydroalcoholic extract in calcium channel modulation was investigated in depolarized rabbit aorta.

Mangla *et al*. (2010) performed an assay on antioxidant activity of methanolic extract of *Curcuma caesia* rhizome. The IC_{50} value of rhizome extract and BHA (Butylated hydroxytoluene) found to be 862.35µg and 46.25µg for 2ml of 500µM concentration of DPPH. This shows the extract with moderate antioxidant activity as compared to BHA. The DPPH radical scavenging activities of methanolic extract of rhizome was

higher in *C. caesia* as compared to *C. amada* (Krishnaraj *et al*., 2010) in dose dependent manner.

Karmakar *et al*. (2011a.) carried out the in vitro antioxidant activity of methanol extract of *Curcuma caesia* rhizome as many diseases are associated with reactive oxygen species. The results obtained with IC_{50} values for scavenging of free radicals were 94.03 ± 0.67µg/ml, 155.59 ± 3.03µg/ml 68.10 ± 1.24µg/ml, 21.07 ± 1.78µg/ml, 260.56 ± 12.65µg/ml and 33.33 ± 0.52µg/ml for DPPH, nitric oxide, superoxide, hydroxyl, peroxynitrite and hypochlorous acid respectively. The reductive ability was shown in dose dependent manner with good potential of antioxidant source in methanol extract.

Karmakar *et al*. (2011b.) assessed on important neuropharmacological activities of *Curcuma caesia* rhizome. There observed significant influence on locomotary activity of mice demonstrating decrease in locomotory activity along with CNS depressant property in mice. The results also revealed that methanolic extract of *Curcuma caesia* significantly antagonized PTZ- induced convulsion showing its anticonvulsant as well as CNS depressant potential.

Kaur *et al*. (2011) compared the analgesic and antipyretic activity of different extracts obtained from *C. caesia* and *C. amada* rhizomes. The activity of extract was evaluated using chemical model of acute pain and brewer's yeast induced hyperthermia in rats. Where, *Curcuma caesia* showed less response as compared to *C. amada*.

Angle *et al*. (2012) isolated the heat stable protein from aqueous soxhlet extraction of rhizome *Curcuma caesia*. Heat stable protein showed significant antioxidant activity when tested on carrageenan rat model system; it also showed high anti-inflammatory activity at dose level of 100mg/kg.

The anti-ulcer activity of ethanolic extract of *Curcuma caesia* in the experimental animal model has been studied by (Das *et al*., 2012). The treatment of rat of either sex was used to evaluate anti-ulcer activity. The significant reduction of ulcer index, gastric acid volume and total acidity along with increased production of gastric mucus in Aspirin induced ulcer animal model was observed with the treatment of EECC (500mg/kg). *Curcuma caesia* extract thus reduced gastric volume and gastric acid secretion in pretreated rat with aspirin.

Antitumor activity of methanolic extract of *Curcuma caesia* against EAC (Ehrlich's Ascites mice) treated mice has been explored by Karmakar *et al*. (2013). The rhizome extract found to be direct cytotoxic (IC_{50} 90.70±8.37µg/ml) on EAC and exhibited significant decreased in tumor volume, weight and viable tumor cell. There was also the attenuation of viability of EAC cells happened due to antioxidant effect of methanolic extract.

Hadem *et al*. (2014) reported the anticancer property of *Curcuma caesia* against diethyle nitrosoamine (DEN) induced liver cancer. DEN induction increased the activities of marker enzymes such as AST (Aspartate aminotransferase), ALT (alanine aminotransferase), ALP (alkaline phosphate), AchE (Acetylcholine esterase), thereby increasing the number of preneoplastic nodules. *Curcuma caesia* thus able to down regulate the activities of marker enzymes prevents depletion of enzymatic and non-enzymatic antioxidant defence system in livers of animals treated with DEN when compared with normal animals.

Devi *et al*. (2015) evaluated antimutagenic property of *Curcuma caesia* rhizome extract against indirect acting mutagen cyclophosphamide. Ethanolic extract reduced the mutagenicity by 97.21% and 90.30% respectively in the *Salmonella typhimurium* strains TA98 and TA100 (in the presence of S9) at its highest tested dose (5000µg/plate) showing strong effective antimutagenicity against cyclophosphamide.

TNF-α induced cell proliferation are mediated by the activation of NF-κB dependent pathways. *Curcuma caesia* lowered TNF-α (Tumor necrosis factor-α) level and NF-kB (Nuclear factor-kB) binding activity in treated mice results in a marked reduction in tumor onset and tumor burden. Hence, it is also supposed to have anti- inflammatory, anti-proliferative and anticancer effects through the TNF mediated NF kB signaling pathway (Hadem *et al*., 2015).

Reenu *et al*. (2015) determined higher levels of DPPH radical scavenging activity in chloroform and methanol with 67.76% and 67.36% and lowest activity was expressed in hexane extract (23.43%) of fresh rhizomes. While, in case of dried rhizomes extract maximum activity was observed with 92.98%, 91.48% and 73.07% in chloroform, ethyl acetate and methanol respectively.

Shakya *et al*. (2015) studied about different fraction of *curcuma caesia* for its antibacterial and cytotoxic activity. The studied revealed the potent cytotoxic effect of petroleum ether extract, diethyl ether extract, chloroform extract and methanol extract showing LC_{50} value of 18.923mcg/ml, 1.086 mcg/ml, 45.289 mcg/ml and 100 mcg/ml respectively, less than 1000 mcg/ml. The antibacterial activity has not shown by any extract because of the absent of tannins in all the extracts of it.

Chadalavada and Budala (2017) studied the anthelmintic activity of three different extracts of *Curcuma caesia* Roxb. based on time taken for paralysis and death of earthworm. The most effective results shown by ethanol extract of (100mg/dl) at 18.06±0.74 and 36.81±1.13 minutes causing paralysis and death of earthworm. Whereas, the standard Albendazole (20mg/dl) showed the results in 23.09±1.56 minutes and 37.20± 1.74 minutes respectively. Hence, ethanolic extract shown to have better vermifuge activity than vermicidal activity as compared to Albendazole in dose dependent manner.

Majumdar *et al*. (2017) explored the anti-hyperglycemic activities of methanolic extract of *Curcuma caesia* rhizome in STZ (Streptozotocin) induced diabetic rats. And, the result showed the effective antidiabetic activity by lowering blood glucose level and controlled intestinal absorption of monosaccharides by inhibiting alpha-amylase and alpha-gulcosidase with IC_{50} 442.92±10.05 µg/ml and 95.40±9.74 µg/ml respectively.

Mahanta *et al*. (2019) identified the four thermolabile sesquiterpene molecular markers from the essential oil of black turmeric. Applicability of 1H-NMR technique for the authentication and phytoformulation of black turmeric and quality control was developed. This developed technique was further employed for drying and storage condition of black turmeric prior to essential oil extraction.

Yadav and Saravanan (2019) assessed the in vitro antioxidant activity of methanolic extract of *C. caesia and C. amada* rhizome against DPPH assay method. They observed higher percentage of inhibition of DPPH free radical in *C. amada* with IC_{50} (63.69µg/ml) than that of *C. caesia* (170.81µg/ml) and the effect was concentration dependent.

CHAPTER THREE: MATERIALS AND METHODS

3.1. Materials

3.1.1. Solvents

Hexane, Chloroform, Methanol, Dichloromethane, were the solvents used in extraction procedures. All of these were of analytical grade and manufactured by Qualigens Fine Chemicals India, HI Media Lab. Pvt. Ltd., India and Merck Ltd, India.

3.1.2. Chemicals used and test organisms

Dimethyl sulfoxide ($DMSO_4$), Ascorbic acid, 2 ─ Diphenyl ─ picrylhydrazyl DPPH, NaCl, Sephadex LH 20, distilled water,

Test organisms- zoological organisms used for this research were **Artemia salina (Biotech. Lab, Central Department of Botany)**.

3.1.3. Equipments

3.1.4. Glass wares

Beaker, conical flask, measuring cylinder, pasture pipette, test tubes, funnel, glass rod, columns, TLC plates, vials, round bottom flasks, seperating funnel, watch glass, Micropipettes, centrifuge tube.

3.1.5. Instruments

Rotatary evaporator and water bath (Eyela, Japan), Grinder mixture (Baltra, India), Dryer, Electronic balance, Refrigerator, UV light for TLC, Spectrophotometer (CT8600, E-chrome Tech, Taiwan), Autoclave.

3.2. Methods

3.2.1. Collection and identification of plant materials

Fresh rhizomes of Black turmeric were collected from the locality of Bahundada, Ghorahi, Dang districts of Nepal during the month of August and September. Identification of sample was done with taxonomist of Central Department of Botany and with various literature review (Press *et al*., 2000). Collected sample then made soil free, washed throughly and shade dried at normal room temperature. Rhizomes were sliced into small cuts for fast dry. Well dried rhizomes were crushed to coarse powder for further steps of extraction.

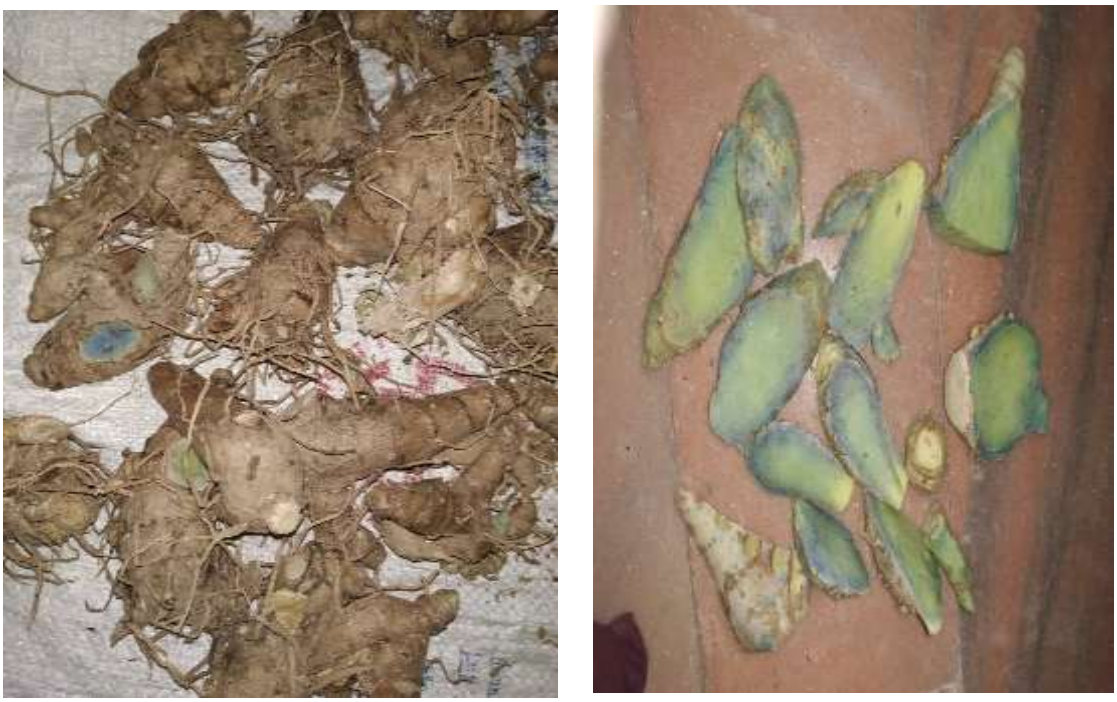


Figure. 2. *Curcuma caesia* rhizomes.

3.2.2. Preparation of rhizome extract

About 100g of (dry weight) rhizome powered was immersed in 500 ml of Methnol:water (9:1 v/v). This setup then kept for 48hrs in dark condition at room temperature with intermittent shaking. Extract was filtered through normal filter paper. The extraction was repeated for two to three times so that extraction of extractable compound would be extracted properly. Finally, filterated solvent was concentrated to

dryness at 35°C- 40°C under reduced pressure by rotatory vaccum evaporator (Eyela) and finally semi solid crude methanolic extracts obtained stored at 4◦C for further steps. Outline for crude methanol extraction given in figure 3.

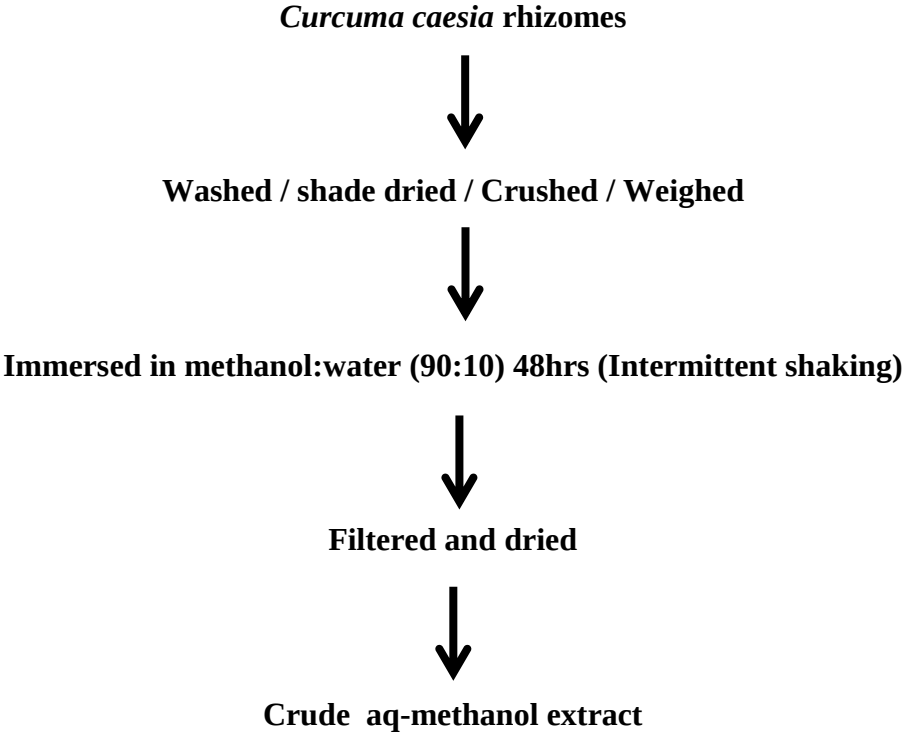


Figure3. Steps for the crude methanol extract from *Curcuma caesia* rhizomes.

3.2.3. Sequential fractionation of crude extract

Sequential fractionation of the crude extracts is one of the most popular method for partial purification (group seperation according to polarity) of crude extract of plant (Eloff, 1998). The Aq-methanolic extract of rhizome was sequentially fractionated with hexane, Dichloromethane and methanol with their increasing polarity as shown in figure 4. Crude extract thus obtained was liquefied with water (100 ml). Hexane (500ml) and liquefied water extract was added in a separating funnel and shaken properly, kept for proper separation into two distinct layers. The lower water layer was taken out and the phenomenon repeated thrice. All hexane fractions washed away fats and pigments following evaporation to obtain hexane fraction. Similarly, the water phase of extract was further washed with medium polar solvent Dichloromethane (DCM) to obtain DCM fraction. Finally, water phase was separated between methanol and water and the respective solvents were evaporated & dried to obtain methanol and

water fractions. Solvents vaporized under reduced pressure at 40°C and finally lyophilized and noted their extractive amounts and kept in air tight glass bottles at -10°C till further use.

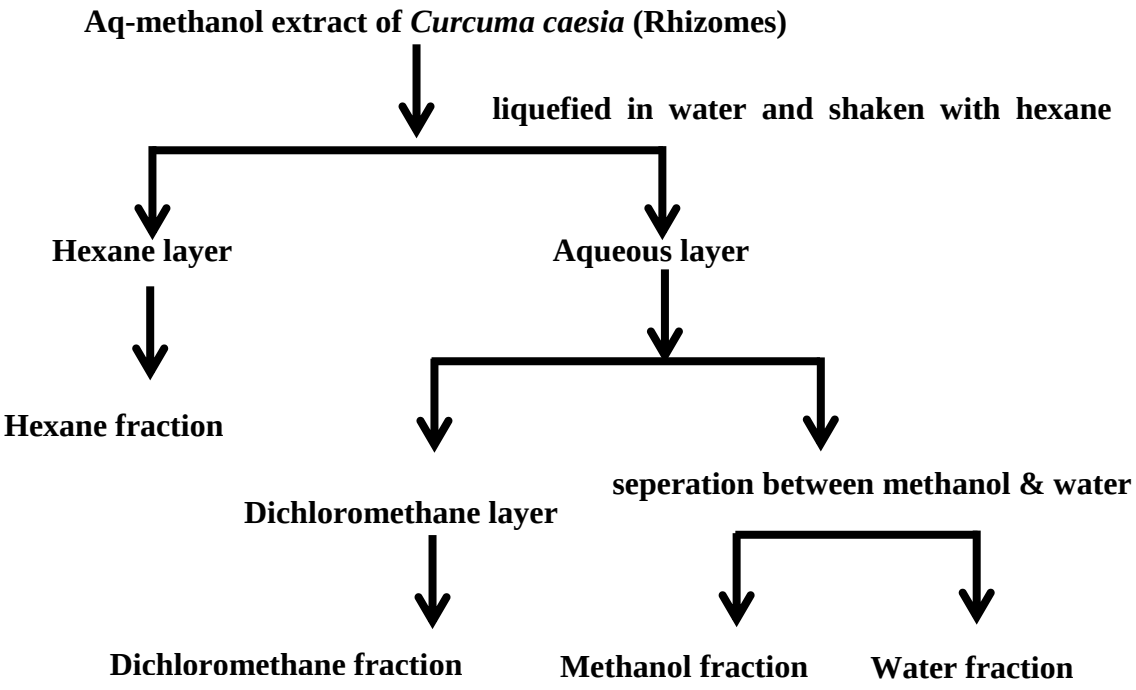


Figure. 4. Steps for sequential fractionation of crude MeOH extract.

Figure.5. Solvent- solvent partioning by Hexane and DCM.

3.2.4. Estimation of extract yields

The mass of dried powder (DP) of sample and total extract (TE) in methanol:water was measured. Then, mass of extract of each fractions (E) was measured after removing its solvents by rotavapour. Similary, percentage of dry extract yield was expressed in percentage using following formula:

a. Total extract yield of dry mass (EYD) = [(TE/DP)× 100]%
b. Yield of each fraction (YOE) = [(E/TE)×100]%

3.2.5. Antioxidant assay

3.2.5.1. DPPH- methanol solution

The DPPH powder 3.943 mg (Mol.wt. 394.32g) was dissolved in methanol 100ml for making 0.1 mM of DPPH methanol solution. Final volume was maintained as per the requirement for the entire test of rhizomes extracts. DPPH solution was freshly prepared for experiments.

3.2.5.2. Rhizomes extract solution

All the three fractions (hexane, DCM, and Methanol) of rhizome extract (10mg) each dissolved in methanol maintaining volume (10ml) separately to prepare the stock solution of 1mg/ml. Then, serial dilution of every extract solution (100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3µg/ml and 1µg/ml) was prepared from stock solution.

3.2.5.3. Reference standard solution

Ascorbic acid (10 mg) was dissolved in methanol (10ml) to prepare stock solution of 1mg/ml, again series of dilution of reference standard compound was prepared by diluting stock solution into series of 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3 µg/ml and 1 µg/ml respectively.

3.2.5.4. DPPH radical scavenging activity

The antioxidant activity was performed by following standard method of (Zhao *et al*., 2006) with some minor modifications. Thus prepared DPPH (3000µl) solution was

transferred into test tubes and extract/ fractions (1000µl) was added individually followed by serial dilutions (1µg/ml to 100µg/ml) to every test tubes and the experiments done in three replicates for each concentration. After that it was kept in dark for 30 min, absorbance was observed at 517nm using spectrophotometer. Ascorbic acid was set as standard positive control of same concentration. Methanol alone was taken as a blank with same volume for each fraction types. Control was prepared with constant volume with and without any extract and ascorbic acid. Percent scavenging of the DPPH free radical was measured by using the following equation:

$$\% \text{ of DPPH scavenging activity} = [1-(A_1-A_2)/ Ac] \text{ X } 100\%$$

Where,

A1 = Absorbance of the extract with DPPH

A2= Absorbance of extracts without DPPH and Ac was the absorbance of control.

Figure 6. Test of extract with DPPH solution.

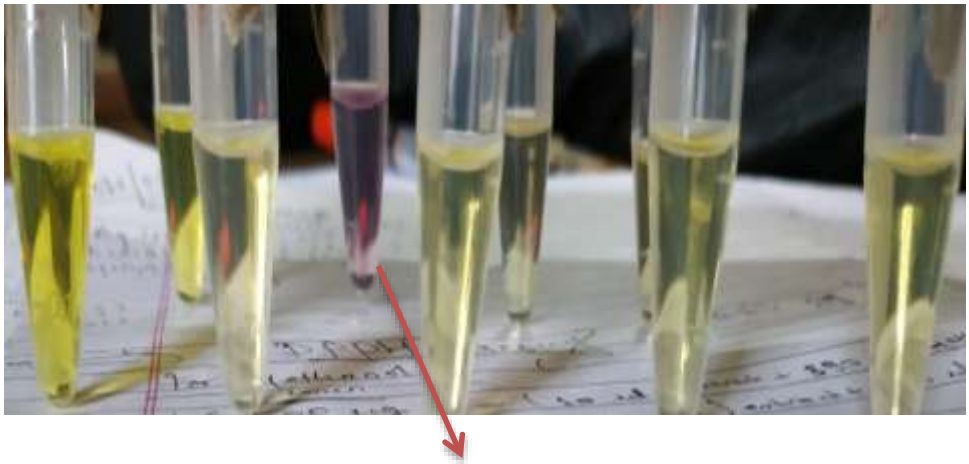
3.2.6. Sephadex - LH 20 Column Chromatography of active (DCM fraction)

Sephadex LH 20 is also called as gel filtration or size exclusion column chromatography in which constituents get separated based on their molecular size. DCM fraction sample of antioxidative extract from black turmeric rhizome was

accurately weighed (550 mg), and loaded into column chromatographic separation of Sephadex LH- 20. The gradient elution with methanol was performed where balanced between elution and flow rates with maximum resolution were maintained.

Flow rate of extract from column though maintained as 5 to 6 drops/min. Separation of layer in column was not so distinct after 4 to 5 layer as shown in figure 7. So the eluents were collected individually in different conical flask with 25 ml each. The total of 9 sub fractions of eluents were collected, performed preliminary qualitative test for antioxidant activity for each of eluents collected using DPPH solution to find active sub fractions (Fig.8). Further, purification of compound was done with TLC profiling. Among different sub fractions, sub fractions 2, 3, 4 and 7 were subjected to TLC with TLC silica gel 60 $F_{254.}$

Figure 7. Sephadex gel filteration of DCM fraction



(Control)

Figure 8. Qualitative test for active fractions against DPPH solution.

3.2.7. Thin layer Chromatography for antioxidant constituents analysis

Gel filtration of active sub fractions after concentrated in amount further subjected to chromatographic profiling. Approximately 100µg of (10mg/ml) extract from each of active sub fractions were prepared for the spotting on the TLC plates (Merck, 10*10 cm^2). The plates were spotted with 5-10 µl of samples and dried with dry stream of air while spotting. The solvent system of 5% methanol in chloroform used as developing system. Thus, prepared plates were air dried and observed under UV and visible light (Fig 9). Then, DPPH solution (2.54 mM) in methanol was sprayed over the plates and incubated in dark for 30 minutes. The active band with yellow (colorless) spots against a purple background showed the presence of free-radical scavenging activity of the spots. Spots were separated by TLC plates in the (5% methanol in chloroform) solvent systems and collected by scrapping silica from the TLC plate for further study. Finally, the movement of active spots (compound) was expressed by retention factor (R_f).

R_f = Distance traveled by solute / distance traveled by solvent

Figure. 9. TLC plate stained with 2.54mM DPPH solution in methanol and visualized under UV light 254nm.

3.2.8. Cytotoxicity Test

A simple bioassay for preliminary assessment of toxicity for natural product research was carried out with brine shrimp which provides easy, quick alternative in case of vertebrate testing. This method is based on aptitude to kill laboratory cultured brine shrimp (***Artemia salina***) (Meyer, 1982). The lethal concentration (LC_{50}) finally determines the bioactivity of test samples against brine shrimp nauplii.

3.2.8.1. Preparation of solution of extracts

Each of three fraction was dissolved in water with addition of DMSO to prepare stock solutions (5mg/ml) of varying concentration (500µl, 100µl, 10µl and 1µg/ml) were obtained by the serial dilution technique and for proper mixing solution was kept in centrifuge at 15000 rpm for 5 minutes.

Artificial sea water was created by dissolving 3.8 g of NaCl into conical flask containing 100 ml of water. Simply NaCl solution in water is taken as control.

3.2.8.2. . Hatching the shrimp

Hatching of brine shrimp was done in flask containing 100 ml of artificial sea water by spreading 18 mg of brine shrimp eggs. The conical flask was illuminated with continuous supply of light for about 24 hours at room temperature 25±1°C to hatch. After 24 hours, naupalii were collected through pasture pipette.

3.2.8.3. Test of extract and brine shrimp nauplii

From the stock solution, 500µg/ml, 100µg/ml, 10µg/ml and 1µg/ml were transferred into twelve vials each with three replicates for three different fractions; hexane, DCM and methanol respectively. Live nauplii were counted in the stem of pasture pipette with magnifying glass against lighted background and active 14 motile larvae were then transfer into each vial and volume maintain as 1 ml in each vial adding artificial sea water. Similarly, same numbers of active nauplii were kept triplicate in artificial sea water only as a control group.

After 24 hours, vials were observed with magnifying glass and the numbers of survival nauplii in each vial were counted and recorded. From the data recorded, the percentage of mortality of nauplii was calculated for each concentration of sample. Thus, mortality rate of nauplii indicates the toxicity of the extract (fraction) or presence of active compound in sample extracts. The medium lethal concentration of (LC_{50}) of the test sample was calculated from best fit line obtain by linear regression analysis of graph. % of mortality can be calculated by using following equation:

$$\% \text{ Mortality} = \frac{N0 - N1}{N0} \times 100$$

Where, N0 = Number of nauplii taken

N1 = Number of nauplii dead

3.3. Statistical analysis

All results were expressed as mean ±SD. The tests were carried out in triplicate form for DPPH free radical assay methods. The percentage of inhibition of DPPH free radical was expressed in mean ±SD. For the determination of inhibitory concentration (IC_{50}),

linear regression equation of concentration Vs scavenging activity plotted in MS excel 2010.

For Brine shrimp toxicity test, all experiments carried in triplicate. Similarly, an approximate linear regression was observed when logarithm of concentration versus percentage of mortality was plotted on the graph. And the values of LC_{50} were calculated by probit analysis (Probit table) statistical methods using Microsoft Excel 2010.

CHAPTER FOUR: RESULTS

4.1. Estimation of yields from dry powder

The rhizomes of *Curcuma caesia* provided with 22.26% of dry powder yield from the freshly weighed (449.27g) rhizomes. While percentage of crude dried extract obtained from powdered materials recorded as 10.73%. The details of dry powder yield and extract yield are presented in annex table1.

4.2. Percentage yield of the fractionated extract

Sequential fractionation of *Curcuma caesia* rhizome extract with three different solvent having different polarity was performed. Physical appearance of Hexane and DCM fractions were yellow and orange red in color, followed by brownish yellow color of methanol fraction. Hexane fraction yielded 25.88% of dried extract from powdered material while methanol fraction yielded 45.23% as lowest and highest yield respectively. In the same way, DCM fraction gave 28.99% of dried extract. It has been observed from the result that, extractable compound of polar nature are found to be higher in rhizomes as the percentage of extract yield was decreasing with the solvent having decreasing polarity (Fig 10.).

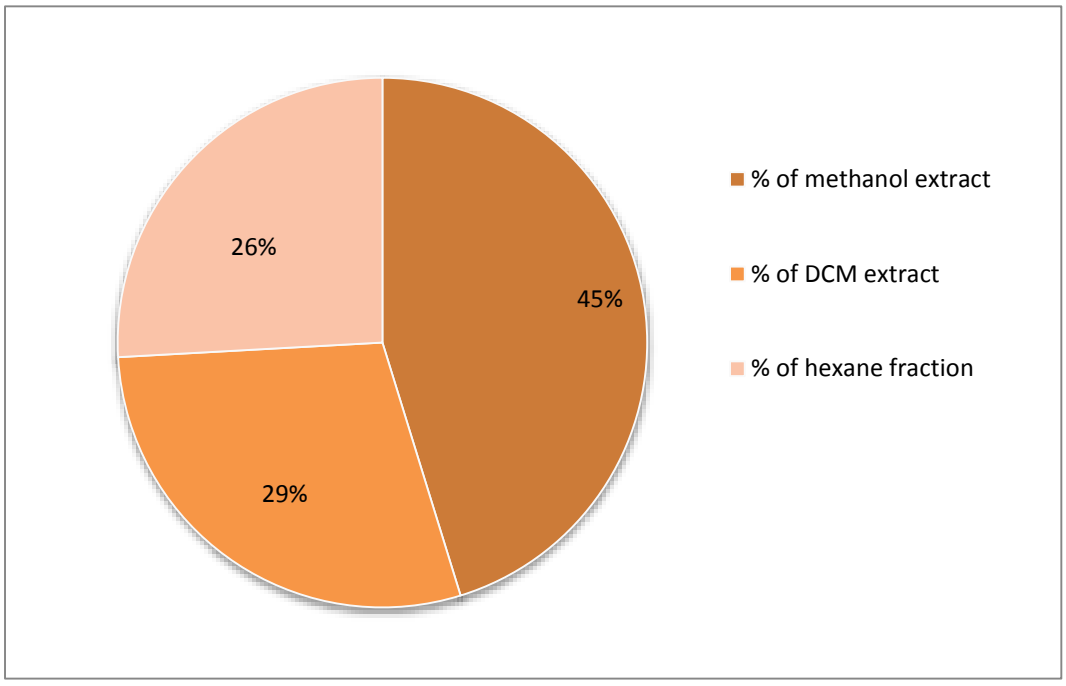


Figure10. Extract yield after solvent-solvent partitioning.

4.3. Antioxidant activity

The antioxidant potential was significantly varying among the extract of different fractions of *Curcuma caesia* rhizome. Ascorbic acid (commercial standard) is strong antioxidant agent, IC_{50} of this compound determined as 8.43±1.28 µg/ml. DCM fraction (IC_{50} 42.72± 1.06 µg/ml) showed high in antioxidant activity while hexane with low activity (IC_{50} 214.87 ±0.78µg/ml) shown in figure11.

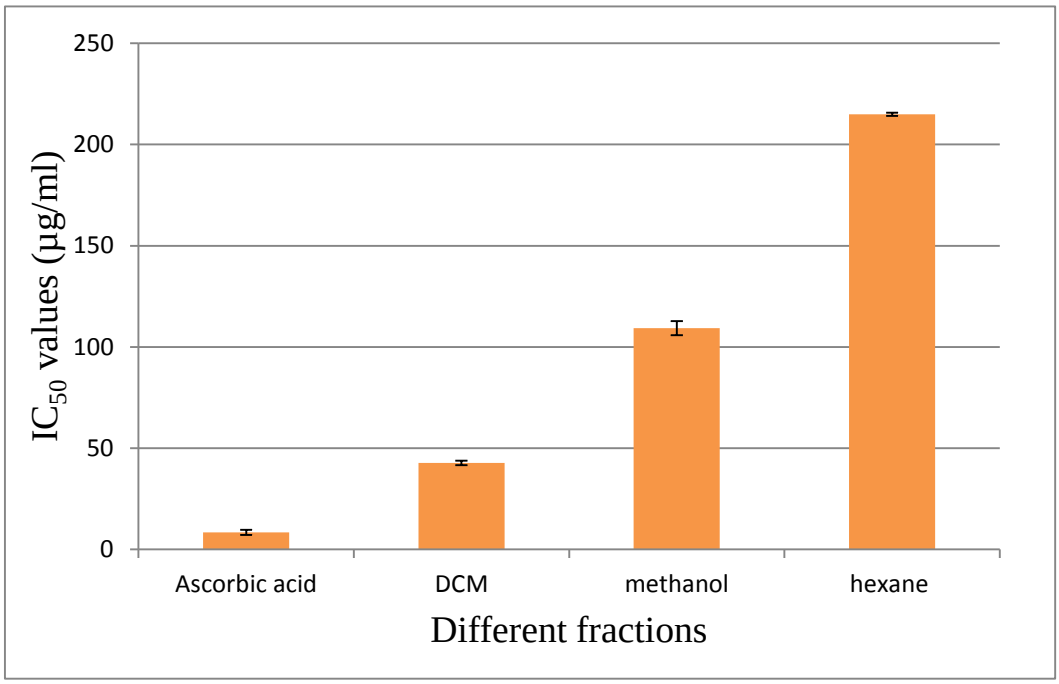


Figure11. IC_{50} values against different fractions of rhizome extract.

4.3.1. Radical scavenging activity of methanol extract

DPPH radicle scavenging activity of methanol fraction of *Curcuma caesia* rhizome was found to be dose dependent (Fig. 12, plot. 1). The highest activity for the DPPH radical scavenging assay was found with (100µg/ml) i.e. 42.57±0.32% and lowest with (1.5 µg/ml) i.e. 2.44±1.5%. It means methanol fraction with 100µg/ml has highest antioxidant activity while that with 1.5µg/ml showed lowest antioxidant activity. Rests of the concentration 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml and 3.125µg/ml were found with 31.68±0.67, 27.70±1.5, 18.70±2.5, 11.51 ±2.03 and 8.30±1.5 percentage of antioxidant activity respectively.

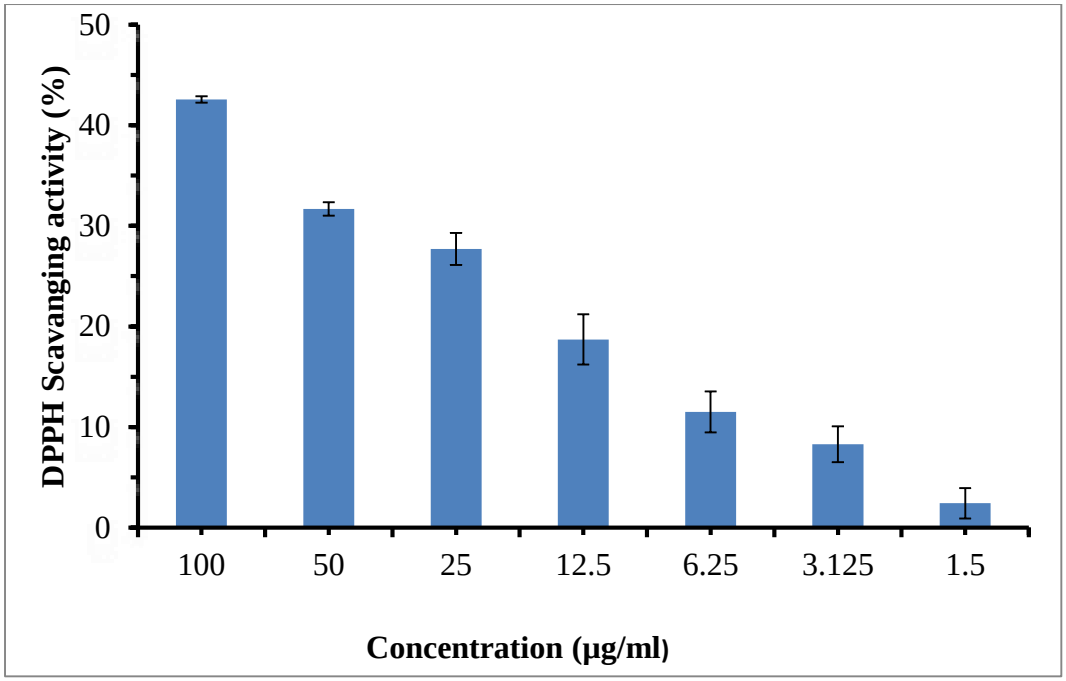


Figure12. Antioxidant activity of methanol fraction against various concentrations

4.3.2. Radical scavenging activity of DCM fraction.

The DCM fraction also inhibited DPPH free radical in dose dependent manner shown effectively from dose- response graph of DPPH radicle scavenging activity assay against varying concentrations (Fig.13).

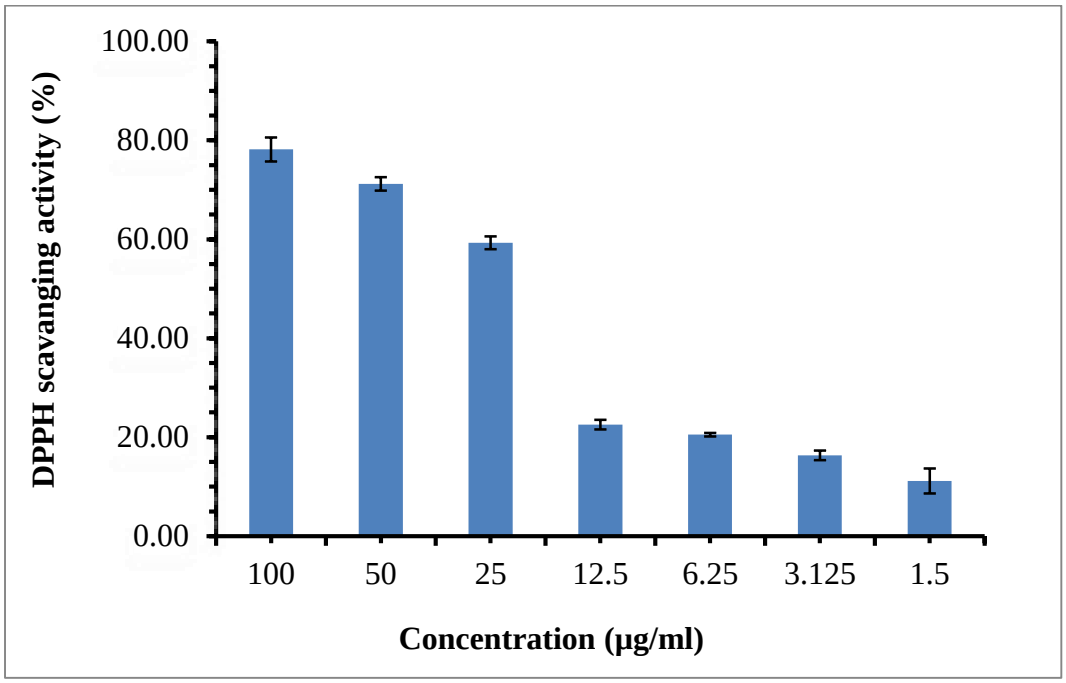


Figure13. Antioxidant activity of DCM fraction against various concentrations.

It was observed that higher inhibition of (78.17±2.47%) at a concentration of 100µg/ml and lowest with (11.15±2.57%) at 1.5µg/ml respectively for this assay. Rest of the concentrations 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml and 3.125µg/ml were found with decreasing order of antioxidant activity; 71.21±1.35%, 59.27±1.28%, 22.52±0.99%, 20.50±0.36% and 16.32±0.96% respectively.

4.3.3. Radical scavenging activity of hexane fraction

Dose dependent DPPH radical scavenging activity of the hexane fraction follows the same trend as that of methanol and DCM fraction (Fig.14). The highest and lowest activity of (23.03±1.03%) and (-3.80±1.57%) was observed at concentration 100 µg/ml and 1.5 µg/ml respectively. 19.61±0.42, 14.65±1.39, 12.14±0.53, 12.07±3.32 and 3.14±1.28 percentages of antioxidant activity was observed in the concentrations 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml and 3.125µg/ml respectively.

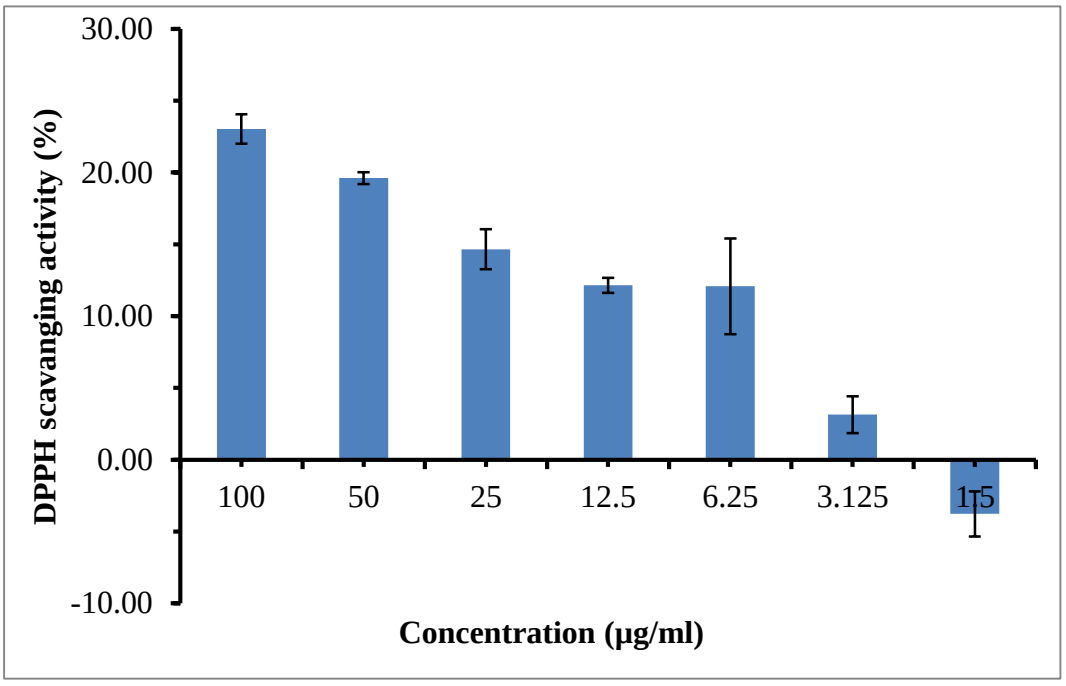


Figure14. Antioxidant activity of hexane fraction against various concentrations.

4.4. Isolation of active compounds using Sephadex LH-20

Using the Sephadex LH-20, it has been possible to isolate or purify active sub fractions having strong antioxidant activity (SA) from the pool of nine DCM column sub fractions. A total of nine sub-fractions (I-IX) were obtained from the DCM fraction of

Curcuma caesia rhizome using sephadex LH-20 with methanol as the mobile phase. Among those, four sub fractions (2, 3, 4 and 7) were found to have more active (strong antioxidant) constituents as compared to the rest of the sub fractions. The category of different sub fractions were observed on the basis of concentration of extract used and time taken to change purple color of DPPH into yellow as shown in table 2. Sephadex purify the compound on the basis of molecular size, so the sub fractions from 2- 4 with high molecular weight compound possess better DPPH scavenging activity. The majority of the sub fractions (1, 5, 6, 8 and 9) were found to possess mild and very least activity. In general, there was no clear trend in antioxidant activity among these sub fractions, but difference in DPPH scavenging activities appeared to be explained only by their relative concentration and time observed.

Table.2. Numbers of fraction isolated or purify from Sephadex- LH-20.

Sephadex fractions.	**Relative concentration (µg/ml)**	**Antioxidant activity**	**Observed time**
Sub fraction 1.	300	LA	Activity shown in 45 minutes
Sub fraction 2.	100	SA	Activity shown within 5 minutes
Sub fraction 3.	100	SA	Activity shown within 5 minutes
Sub fraction 4.	100	SA	Activity observed 10 minutes
Sub fraction 5.	200	MA	Activity observed in 30 minutes
Sub fraction 6.	200	MA	Activity observed in 30 minutes
Sub fraction 7.	100	SA	Activity shown in 10 minutes
Sub fraction 8.	300	LA	Activity observed in 35 minutes
Sub fraction 9.	300	LA	Activity observed in 45 minutes

Where, SA= strong antioxidant; LA= least antioxidant and MA= mild antioxidant on the basis of concentration and time taken to change the color of DPPH.

4.5. Isolation of TLC guided antioxidant compound

All the active sub fractions of gel filtration have been subjected into TLC plates. As expected, the sub fractions showed a numbers of migrated spots having strong intensities on TLC plate. The number of yellow spots, their migration and R_f values were various among different active sub fractions (2, 3, 4, 5and 7). TLC chromatogram of sub fraction (2) showed four active bands (R_f 0.22, 0.34, 0.41 and 0.52) with very fast DPPH scavenging activity. Three bands of sub fractions (3) and (4) with similar range of R_f values (0.25, 0.41 and 0.56) proven to have identical compound with fast antioxidant activity. Similarly, sub fraction (5) also have similar active compound (R_f 0.56) with that present in sub fraction 3 and 4, as it showed only one band. Finally, only one band in sub fraction (7) (R_f 0.46) showed good active spot with potent antioxidant activity. Samples/ sub fractions were compared with one another with regards to number of active spots, relative position (R_f), color and intensity of the spots in given TLC plate (Table 3, Fig 15).

Table.3. Compounds with their R_f values in different sub fractions of black turmeric rhizomes.

Sephadex fractions	**Number of active spots**	**R_f values**	**Reaction speed**	**Intensity of spots**
Sub fraction2	4	0.22, 0.34, 0.41 and 0.52	Fast	+++
Sub fraction3	3	0.25, 0.41 and 0.56	Fast	+++
Sub fraction4	3	0.25, 0.41, 0.56	Fast	++
Sub fraction5	1	0.56	Slow	+
Sub fraction 7	1	0.46	Moderate	++

+, weak; ++ intermediate; +++, strong.

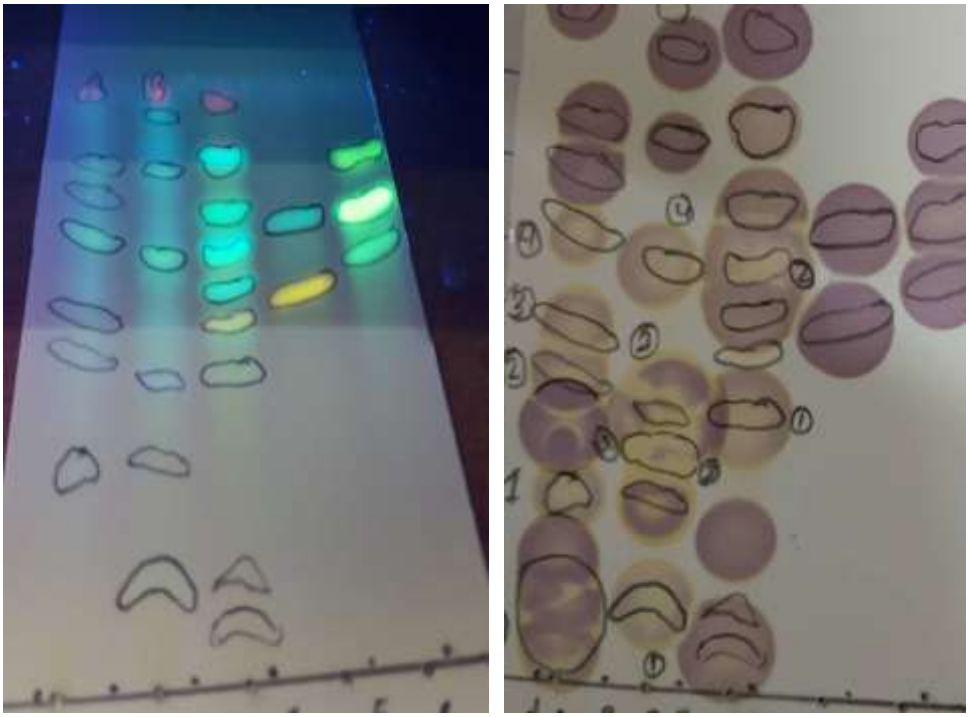


Figure15. TLC of DCM sub fractions stained with DPPH.

4.6. Brine shrimp lethality bioassay

As a cytotoxicity assessment tool, brine shrimp lethality bioassay was used as convenient method and can provide possible to toxicity of the test samples. The results for probit analysis of brine shrimp lethality test for each of the fractions sample are given in figure 15 (Graph a to c).The percentage of mortality of brine shrimp was found to be directly proportional to the concentration of all three solvent fractions (Methanol, DCM and Hexane). The highest percentage of mortality was observed at the concentration of (100µg/ml) in methanol fraction (100%) followed by DCM (97.61%) and hexane (85.71%) respectively. Similarly, percentage of mortality was found to be lowest at the (1µg/ml) concentration i.e. 9.53% which was similar for both methanol and DCM fractions, while hexane fraction showed 21.42% of lethality in the same concentration. Overall, result of different solvents fractions shows that as the concentration increases the lethality percentage also increases.

The parameter used to show the cytotoxic behavior is the value of lethal concentration (LC_{50}). The LC_{50} values obtained from brine shrimp lethality assay (Tables 4) shows higher level of cytotoxic activity in methanol (11.42µg/ml) and hexane (14.12µg/ml) fraction. Interestingly, DCM fraction of rhizomes had minimal toxicity (37.12µg/ml).

Again, all the fractions tested showed good brine shrimp larvicidal activity, resulting LC_{50} values less than 250µg/ml were considered significantly active.

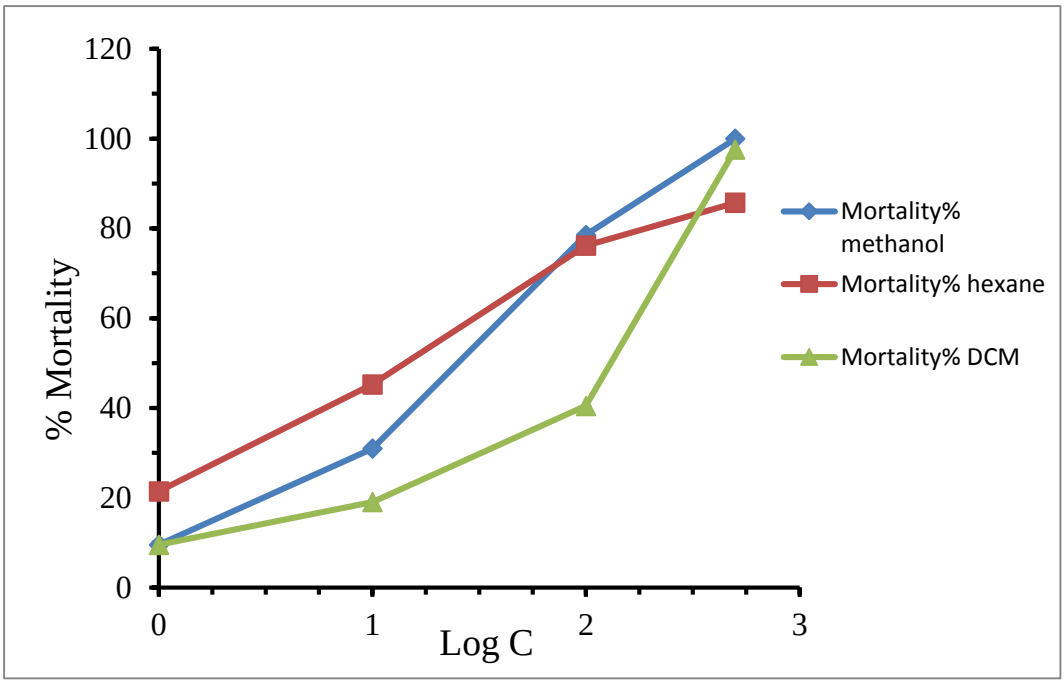
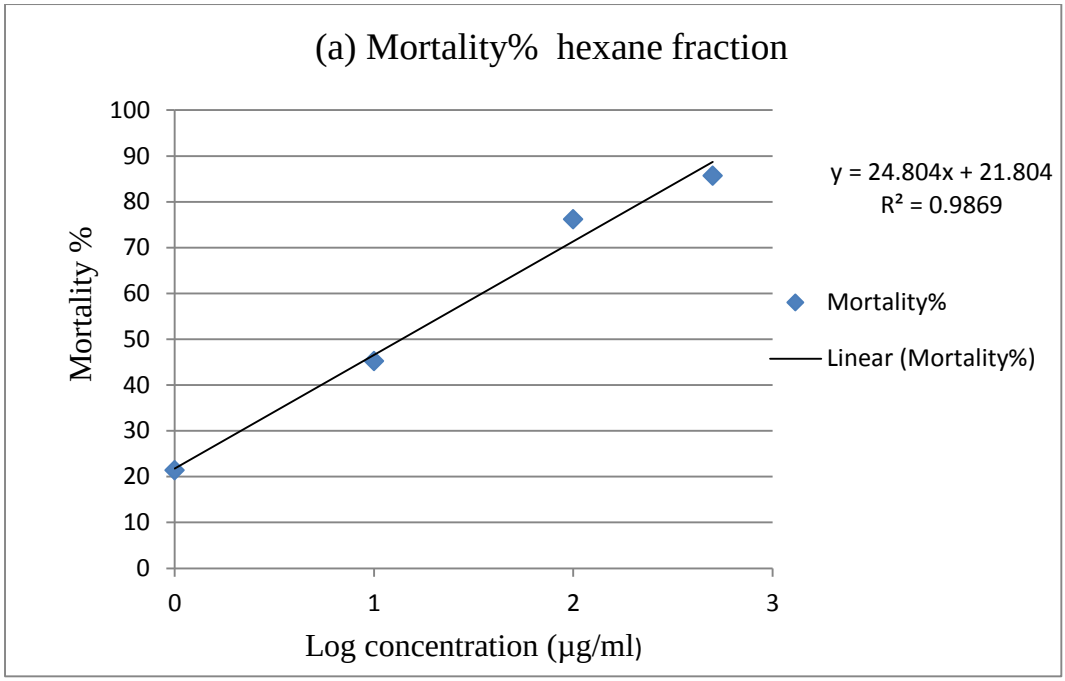
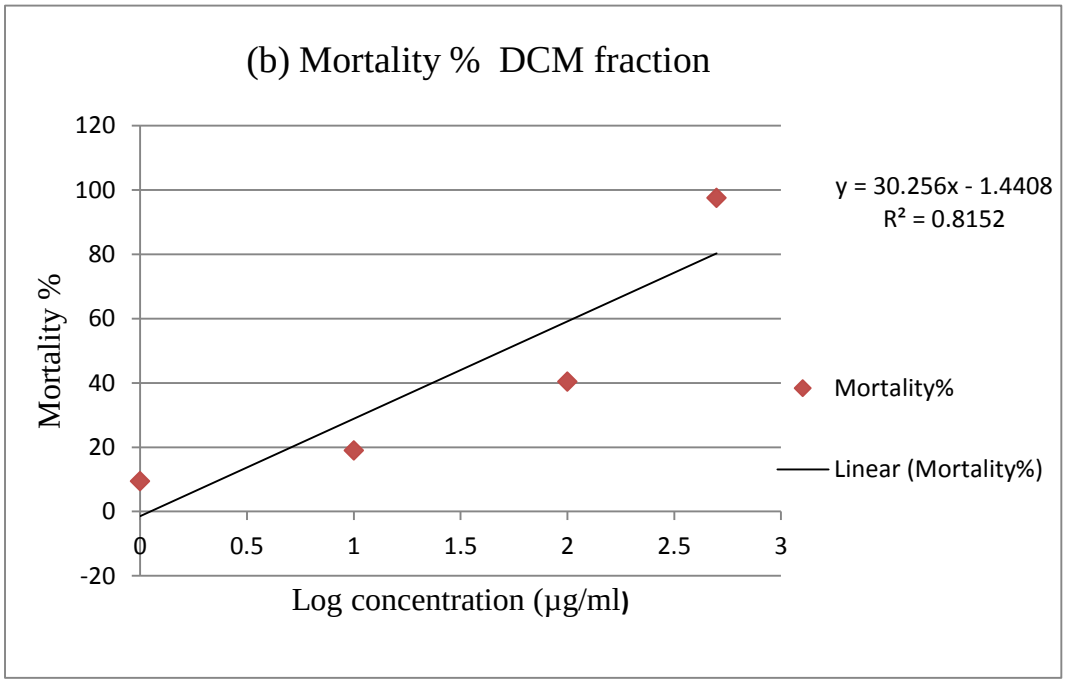


Figure16. Plot of log concentration of n-hexane (— □ —), methanol and (— ◊—), DCM (— Δ —) fraction crude methanolic extract versus percent shrimp mortality after 24 h of exposure.

Table.4. LC_{50} of the different fractions brine shrimp lethality bioassay.

Samples	**LC_{50} (µg/ml)**	**Regression equation**	**R^2**
Methanol fraction	11.22	y=1.54x+3.37	0.92
Hexane fraction	14.12	y=0.70x+4.19	0.99
DCM fraction	37.15	y=1.08x+3.30	0.82


(a) Mortality% hexane fraction
Mortality %
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
Log concentration (µg/ml)
y = 24.804x + 21.804
R² = 0.9869
Mortality%
Linear (Mortality%)


(b) Mortality % DCM fraction
Mortality %
120
100
80
60
40
20
0
-20
0
0.5
1
1.5
2
2.5
3
Log concentration (µg/ml)
y = 30.256x - 1.4408
R² = 0.8152
Mortality%
Linear (Mortality%)

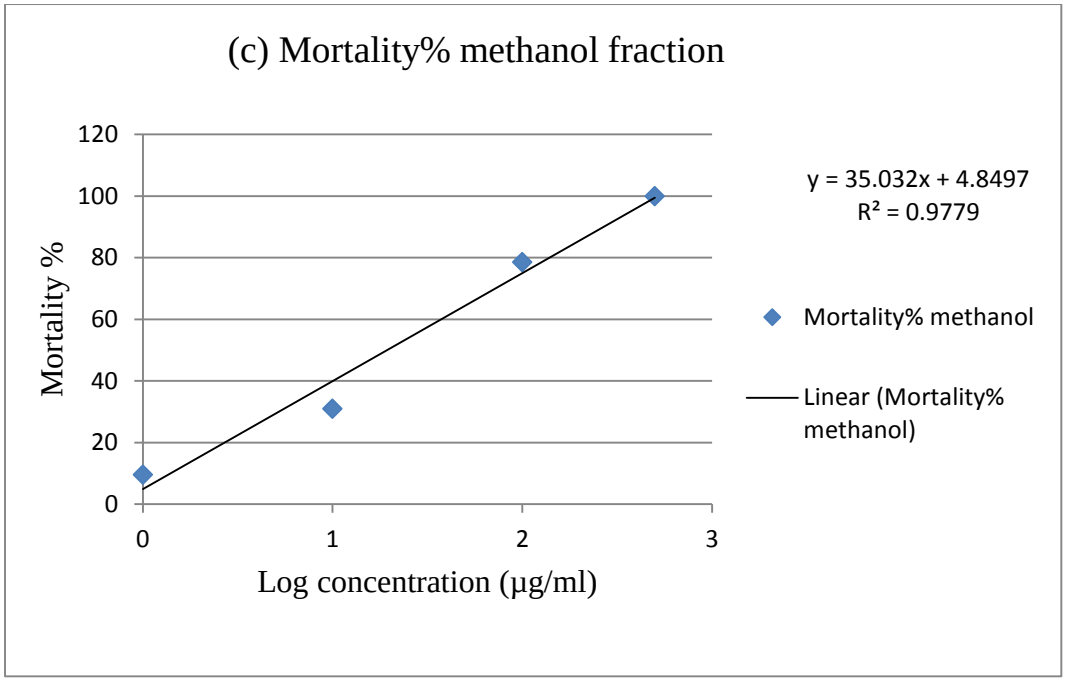


Figure17. Linear regression equation for (a) hexane (b) DCM and (c) methanol fractions to obtain LC_{50} value.

CHAPTER FIVE: DISCUSSIONS

5.1. Extraction yields

The extraction yields depend on both the extraction procedures and the type of solvent used during the extraction. Medicinal plants have a wide range of phytochemical that is directly dependent on the plant´s developmental stages, parts and solvents used for extraction and isolation of phytochemicals (Ullah *et al*., 2017). Most efficient plants parts in term of crude extract quantity is branches (Wakeel *et al*., 2019) and plant parts follows the order of branches > leaves > flower> Roots (rhizomes) on the basis of extract yield. The rhizomes of *Curcuma caesia* was sequentially extracted with hexane, dichloromethane and methanol. The yield of extract differed with the variation of extracting solvents used (Fig.5), ranged from 25% to 45%, maximum yield being shown in the methanol fractions. The purpose of employing sequential extraction with the same powder was to ensure the complete extraction of wide polarity range of compounds. Besides, the chemical components of extractable constituents in plant also influence the extraction efficiency of solvents (Sultana *et al*., 2009).

Based on the results obtained, higher efficiency of extraction was observed in highly polar solvents methanol (45.23%) in dried rhizome as compared to the rest of solvents. This indicates that phytochemicals present in the rhizomes of *Curcuma caesia* are mostly polar in nature.

5.2. Identification of antioxidant activity.

Attention on oxidative stress treatment has been considered widely in recent medicinal practices. Though, remedies of illness with an indigenous knowledge by the use of medicinal plants may be an ancient technique. Such plants based phytocompounds are easily metabolized inside the human body without any detrimental effect that leads to the phytochemical based remedies (Sangwan *et al*., 2002). The antioxidant properties of phytochemicals are linked to their ability to scavange free radicals generated either by endogenous or exogenous agents. The mechanism that contributes to the antioxidant capacity induced by phenols and flavonoids which include free radicals scavenging ability, hydrogen or electron donation ability, chelation of redox reaction active metals ions and interaction with cell signaling pathways (Devi *et al*., 2015; Arouma, 2003).

Therefore, the present study examined the rhizome extract of *Curcuma caesia* Roxb. for antioxidant activity by DPPH free radicle scavenging assay. Three different solvent fractions from crude methanol extracts showed variation in radical scavenging assay among each other as in the dose dependent manner and was significant as in the previous studies (Yadav and Saravanan, 2019; Karmakar *et al*., 2011a). The present assessment also showed DPPH scavenging activities in the order of DECC>MECC>HECC with their percentage of inhibition ranging from -5.30% to 78.15%. The variation occurred with highest antioxidant in dichloromethane (DCM) fraction (IC_{50} 42.72±1.49µg/ml) to the lowest activity in the hexane fraction of *Curcuma caesia* rhizome (IC_{50} 214.87±12.31µg/ml). According to Phongpaichit (2007) extract with IC_{50} 10-50 µg/ml considered to be strong antioxidant activity and that with <1000 µg/ml as weak. The current study showed antioxidant activity below IC_{50} 1000 µg/ml, while even better result with strong antioxidant potential below 50µg/ml has been shown by DCM fraction (IC_{50} 42.72±1.49µg/ml).The results concluded that higher antioxidant activity is present in the samples extracted with semi polar (DCM) solvents.

Various research works with minor differences have been published with the similar result like this assessment in so many other medicinal plants. The presence of antioxidant activity seen from the decrease of absorption values of DPPH radical caused by the test samples at various concentrations and the increasing value of the percentage of inhibition with more samples introduced. The similar trend and results of antioxidant activity has been shown by methanol extract/ fractions of Sanggani leaves (Morjoni and Zulfisa, 2017). *Curcuma* plant contains many bioactive compound such as phenols, flavonoids, essentials oils, starch, sterols and carbohydrates ((Krishnaraj *et al*. 2010; Reenu *et al*., 2015; Mukunthan *et al*., 2017; Paliwal *et al*., 2011). The presence of polyphenols and flavonoid compound are reported to have direct relation with antioxidant potential (Mangla *et al*., 2010; Sarangthem and Haokip, 2012). Similarly, antioxidant potential of methanol fraction of *Curcuma caesia* was with IC_{50} nearly analogous to the methanol extract of same species (IC_{50} 94.03 ±0.67) for DPPH assay of previous study (Karmakar *et al*., 2011) but the antioxidant potential for nitric oxide (NO) and Peroxynitrite (PO) activity had IC_{50} well above it. Reductive ability of the extract was also tested where dose dependent reducing capability was observed. However, the activities of antioxidant have been attributed to various mechanisms such

as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging. Krishnaraj *et al*. (2010) reported that DPPH free radical scavenging activity of *Curcuma caesia* rhizome extracts was higher than *Curcuma amada* rhizome extract (data not shown). Mangla *et al*. (2010) suggested that methanolic extract of *curcuma caesia* with moderate antioxidant potential having IC_{50} 862.35µg for 2ml of 500µM DPPH which is nearly double the IC_{50} of hexane (214.87±12.31 µg/ml) fraction in present study. Because the specificity and sensitivity are different for each used method, it can be concluded that the same antioxidant samples exhibit different antioxidative values depending on the concentration and the measured antioxidant parameter.

Plant having IC_{50} below 50µg/ml considered to be strong antioxidant potential with high level of radical scavenging assay. The higher antioxidant activity of any plant is accredited to various pharmacological properties. In this research, DPPH free –radical scavenging assay was performed to determine antioxidant potential of different fraction of black turmeric rhizomes extract. DPPH test which is based on the ability of DPPH to decolorize in the presence of antioxidant is the direct and reliable method of determining antioxidant activity of compounds present in the plant extract (Hasan *et al*., 2009).The activity of all three fractions of black turmeric rhizomes are recorded in this study had higher and lower antioxidant activity with substantial margin than many other medicinal plants and *Curcuma* species of former studies. Therefore, medicinal property of this *Curcuma caesia* may be of great relevance in the prevention and therapeutics of free radical mediated diseases.

Additionally, it has been observed that oxidative stress now appears the fundamental mechanisms underlying a number of human neurologic and other disorders such as viral infection, digestive disorder and ulcers (Aruoma. 2003). In case of the cancer, ROS is responsible for initiating the multiple stages of carcinogenesis which leads to progressively deregulation of the cell growth and carcinoma. Various epidemiological and laboratory studies demonstrated that antioxidant have substantial protective effect on (cytotoxicity) human carcinogenesis (Tsao *et al*., 2004; Mehta and Pezzuto, 2002). The present research results suggest that all tested rhizome extracts fractions have moderate to potent antioxidant activity. Their antioxidant potential likely contributed to their carcinoma protective effect and their other medicinal usage as the fractions

shows larvicidial effect against brine shrimp napaulii. It is to be clear that antioxidant properties and cytotoxic effect of rhizome extract fraction are distinct and complementary. And their combined use might be synergistic and more effective in the therapeutic drugs industry.

5.3. Purification (Size exclusion chromatography) and isolation using TLC based DPPH assay

The potential antioxidant activity was found higher in DCM fraction of black turmeric rhizomes, which was subjected to isolate and purify its active compound through size exclusion chromatography (Sephadex LH 20). Size-exclusion chromatography is considered a kind of partition chromatography, which is able to isolate compounds according to various molecular sizes. Purification of fractions/extract based on their molecular size of the potent antioxidant showed that there were four active subfractions (Table 1). The other five sub fractions could be very low amount of antioxidant compound which need to be further studied. Sephadex fractions eluted in this study showed strong antioxidant assay in terms of concentration and reaction time observed during experiments. Eventually, it was found that strongly inhibitory effect on DPPH free radical assay of isolated subfractions (2, 3, and 4) presented in table.1. This technique was effective for obtaining the antioxidant compound on a large scale. The sephadex fractions could further applied to determine the purity and variety of compound with TLC.

TLC profiling of DCM sub fractions (Sephadex fractions) gave an impressive result in terms of antioxidant activity that directing towards the presence of number of phytochemicals. Thin layer chromatographic profiling is a vital parameter of herbal medicine calibration for the proper documentation of medicinal plants. Cucuminoids (curcumin, de-methoxycurcumin and bis-demethxothycurcumin) and volatile oil components from rhizomes and leaves of *Curcuma* are considered to be important biologically. Infact, these curcuminoids plays considerable role in bioprotective activity (Sasikumar, 2005). The retardation factor (Rf) value of standard curcuminoids (Himedia) were 0.95, 0.85 and 0.54 respectively. While Revathy *et al*. (2011) showed better resolution of R_f as 0.75, 0.55 and 0.27 for curcumin, de-methoxycurcumin and bis-demethoxycurcumin respectively. Moreover, TLC analysis of *Curcuma caesia*

done by Sarangathem and Haokip (2010) presented R_f values of three compound as 0.42, 0.14 and 0.08 in crude extract than in standard R_f values were 0.39, 0.18 and 0.06 in chloroform and methanol (95:5) ratios as mobile phase. In the present research, maximum resolution of compound showed in sub fractions (2, 3 and 4) provided retardation value (Rf) were 0.22, 0.25, 0.41, 0.34, 0.52 and 0.56 respectively. The entire documented compound showed very potential antioxidant activity at concentration 100µg/ml. This presence of compound with different R_f values were observed to be nearly same in former studies (Sarangathem and Haokip, 2010: Revathy *et al*., 2011). Therefore, there could be the presence of curcuminoids compound along with some volatile oils.

Various phytochemical compounds in sub fractions gave different R_f values (Table 2) ranging from 0.21 to 0.56 with maximum four spots in subfraction (2) followed by three in sub fractions (3 and 4) each. This variation in retardation factors (R_f) simply provides the important information about the polarity and selection of appropriate solvent system for the identification of phytochemical in separation process. Therefore, chromatographic fingerprints would be considered to evaluate the quality of herbal medicine globally considering multiple constituents present in the herbal medicines.

5.4. Cytotoxicity assay with brine shrimp assay

Brine shrimp lethality assay is a convenient method for general screening of toxicity of extracts or compound towards the brine shrimp (Meyer *et al*., 1982, Zhao *et al*., 1992), and this could give an indication regarding cytotoxicity of the test samples. Most of the extracts showed positive results in this assay except a few indicating a strong cytotoxic ability which might have significant role in killing the cancerous cells. LC_{50} value simply determines the level of toxicity in the given extract. And LC_{50} value <1mg/ml is considered to be significant and toxic, while that with > 1mg/ml indicate tested sample to be non-toxic (Meyer *et al*., 1982). However, in this assay, as shown in figure 16, both methanol and hexane fractions showed strong cytotoxicity as compared with dichloromethane. The activity was concentration dependent. Although, brine shrimp lethality assay (BSLA) is insufficient in determining the mechanism of action of the bioactive substances in plants or it's specificity for antitumor activity, it provides a preliminary screen that can be supported by a more specific bioassay, once the active compounds have been isolated.

Result of present assessment revealed that the degree of lethality was found to be directly proportional to the concentration of the extracts as shown in figure16 (graph.a. Graph.b. and graph.c). It has been observed that variation in the toxicity occurs based on the solvent used for extraction which was consistent with the formerly reported studies (Nguta *et al*., 2012). All three fractions of *Curcuma caesia* rhizome showed the high level toxicity towards brine shrimps effect (LC_{50} 11.22 µg/ml to 37.15 µg/ml), meanwhile these values are less than 1000 µg /ml. Methanol and hexane fraction of black turmeric exhibited high level of brine shrimp lethality (LC_{50} value of 11.22µg/ml and 14.13 µg /ml), that may be attributed in part to powerful cytotoxic and probably anticancerous compound associated with the methanol and hexane fraction. Similarly, the hexane fraction of black turmeric have exhibited dose dependent inhibition only in the cancer cell lines (HepG2) with very low (0.976µg/ml) IC_{50} value in the previous study (Mukunthan *et al*., 2017). Devi *et al*. (2015) have reported the chemo preventation role of antimutagenic compound present in the rhizome extract of *Curcuma caesia*. Earlier, Das *et al*., (2012) also purposed the antiulcer activity in aspirin induced gastric ulcer model in animal. The phytoconstituents like flavonoids, saponins and alkaloids (Donipat and Sreeramulu, 2015) in the species have been reported among the cytoprotective active materials for which antiulcerogenic efficacy have been extensively confirmed. Additionally, camphor, alkaloids, sesqueterpenes, monoterpenes and lactones present in the hexane extract of black turmeric rhizomes (Mukunthan *et al*., 2017)), classified as natural compound which were proven to be effective and safe in cancer treatment and management (Huang *et al*., 2012; Rasul *et al*., 2012). However, further investigations using carcinoma cell line are necessary to isolate the active compound(s) responsible for the activity. The positive response obtained in this assay suggests that the extracts may have bioactive toxic compounds.

Furthermore, the antioxidant and general cytotoxic activity of three different solvent extracts fraction found in this study, may explain some of the indigenous medicinal practices to treat snake/scorpion bite, anthelmintic, leprosy, leucoderma and soon (Amalraj *et al*., 1989, Saikia and Borthakur, 2010) from this plants. The brine shrimp lethality bioassay is found to have a good correlation with cytotoxic activity in some human solid tumors and with pesticidal activity and led to the discovery of new class of natural pesticides and active antitumoral agents (McLaughlin, 1998). Therefore, the cytotoxicity of the all three solvent extracts fraction of Black turmeric rhizomes could

be of particular interest in relation to the isolation and identification of new ‘principal’ compounds for the development of drugs against cancer cells, pesticides and so on.

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

This research summarizes the variation in antioxidant activity along with the cytotoxicity of *Curcuma caesia* rhizome extract fractions. The antioxidant activity was determined by DPPH free radical scavenging assay and significantly varied among the different solvent fractions with highest activity in rhizome extract of DCM fraction (IC_{50} 42.72±1.06 µg/ml) and lowest activity in Hexane fraction (IC_{50} 214.87 ±3.49µg/ml). Due to the highest activity of DCM fraction in DPPH free radical scavenging assay, it was further subjected to Sephadex-LH 20 column chromatography to isolate the active antioxidant compound in pure form with their respective R_f values (0.21, 0.22, 0.25, 0.34, 0.41 and 0.56). Moreover, in brine shrimp lethality assay, highest degree of lethality observed in methanol fraction (LC_{50} 11.22 µg/ml) followed by hexane (14.12 µg/ml) and DCM fraction (37.15 µg/ml) respectively. The findings from this study are promising but preliminary in nature. This study also suggests that *Curcuma caesia* rhizome extract could be considered as a significant source of natural antioxidant as well as a potential anticancerous compound.

6.2. Recommendations

Some of the commandments which are drawn from the present research works are:

- ✓ Since rhizome of *Curcuma caesia* possess strong antioxidant potential, characterization and structural elucidation of the antioxidant compounds from these extracts is recommended.
- ✓ Strong larvicidial effect of extract against brine shrimp napaulii also demands the structural elucidation of cytotoxic compounds from this plant, which might be helpful to discover promising molecules for anticancer treatment.
- ✓ A further research is strongly recommended regarding its other parts including rhizome which may deliver useful information in relation to different human health concerns.

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ANNEX 1

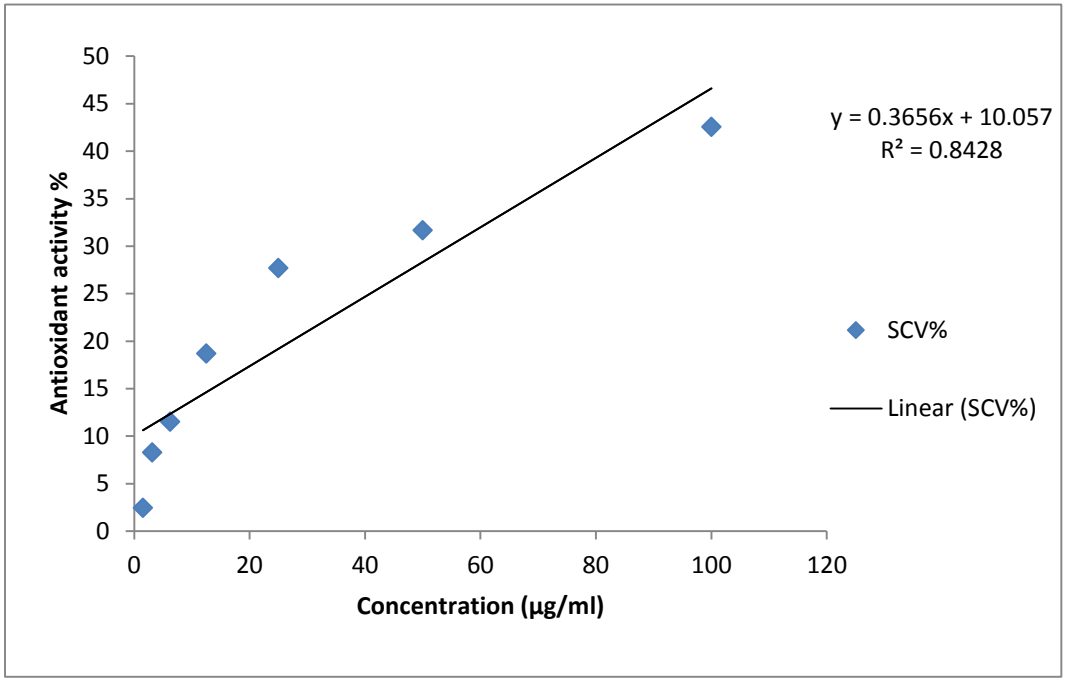
Table.1. Estimation of extracts yield.

Wt. of fresh rhizome(g)	449.27
Wt. of dry powder(g)	100
Wt. of dried crude extract (g)	10.743
% of Dry powder yield	22.26%
% of extract yield	10.74%
% of methanol extract	45.23%
% of DCM extract	28.89%
% of hexane fraction	25.88%

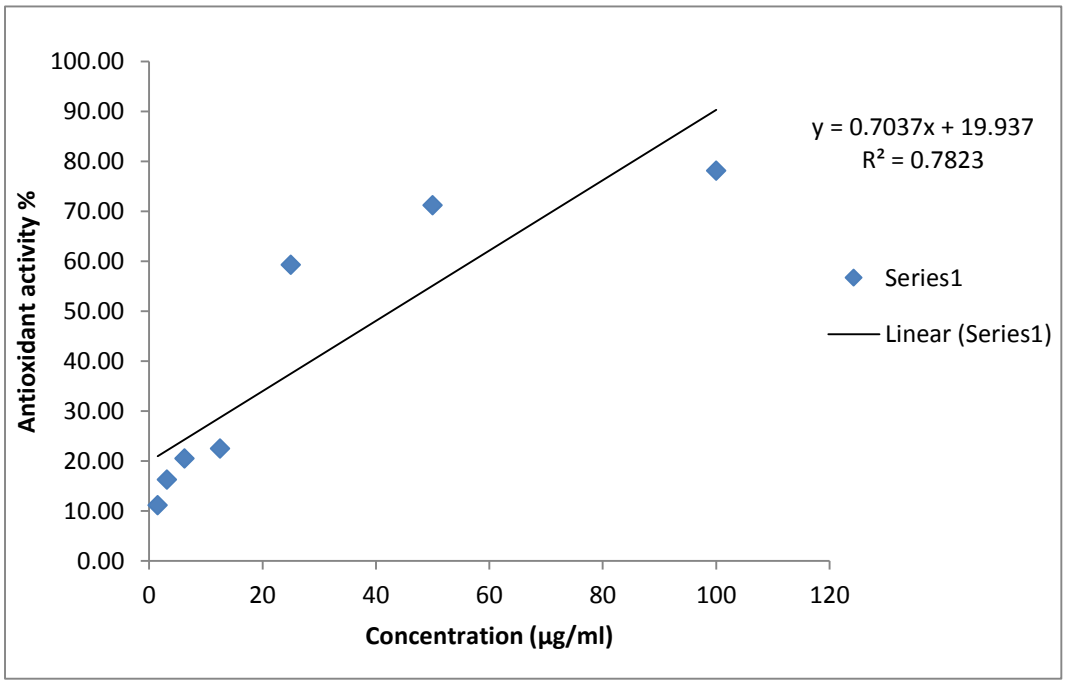
Table.4. Antioxidant activity of different fraction of *Curcuma caesia* rhizome extract.

Antioxidant activity of extract/ fractions (%)								
Concentration(µg/ml)	**Methanol fraction**		**DCM fraction**		**Hexane Fraction**		**Ascorbic acid**	
100	42.23	42.57±0.32	80.96		24.21	23.03±1.03	88.86	
100	42.64		76.57		22.33		87.92	
100	42.84		76.99	78.17±2.42	22.54		88.11	88.29±0.50
50	30.91	31.68±0.67	72.18		19.19	19.61±0.42	79.42	
50	32.17		69.67		19.60		82.25	
50	31.96		71.76	71.20±1.35	20.03		79.80	80.49±1.54
25	28.82	27.70±1.58	58.16		13.96	14.65±1.39	77.53	
25	28.40		59.00		13.75		61.11	
25	25.88		60.67	59.27±1.28	16.26		74.13	70.93±8.67
12.5	17.00	18.70±2.5	23.64		12.71	12.14±0.53	53.31	
12.5	21.49		21.76		11.65		58.65	
12.5	16.68		22.18	22.52±0.99	12.07		53.37	55.44±2.82
6.25	13.75		20.71		9.56	12.07±3.32	39.96	
6.25	9.77	11.51±2.03	20.08		15.84		56.20	
6.25	11.03		20.71	20.50±0.36	10.82		55.63	50.60±9.22
3.125	9.98		15.27		4.54	3.14±1.28	34.11	
3.125	6.42	8.30±1.79	16.53		2.04		40.15	
3.125	8.51		17.15	16.32±0.96	2.86		40.15	38.14±3.49
1.5	3.70	2.44±1.5	8.79		-5.30	-3.80±1.57	35.43	
1.5	0.77		10.88		-3.84		21.27	
1.5	2.86		13.81	11.16±2.52	-2.16		27.31	28.01±7.11
IC50 value ((µg/ml))	**109.25±3.49**		**42.72±1.06**		**214.87±0.78**		**8.43±1.28**	

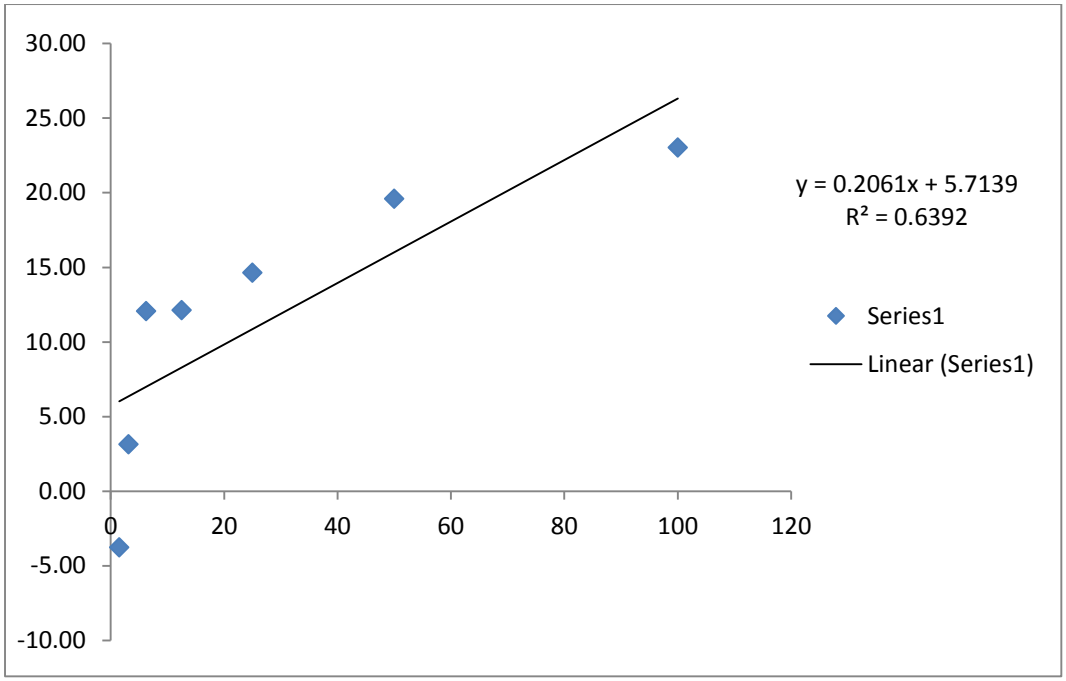
Figure.18. Antioxidant activity (%) against concentration of various fractions for the calculation of IC_{50} values.



Plot.1. Antioxidant activity of methanol fraction.



Plot.2. Antioxidant activity of DCM fraction.



Plot.3. Antioxidant activity of hexane fraction.

Table.5: Effect of n-hexane, methanol and DCM fraction of crude methanolic extract on brine shrimp.

Concentration (µg/ml)	**Log C**	**Mortality% methanol**	**Mortality% Hexane**	**Mortality% DCM**
500	2.698970004	100	85.71428571	97.61904762
100	2	78.57142857	76.19047619	40.47619048
10	1	30.95238095	45.23809524	19.04761905
1	0	9.523809524	21.42857143	9.523809524

Table.6 . Percentage of mortality of Brine shrimp against various fractions of *Curcuma caesia* rhizome extract.

Concentration(µg/ml)	**Log C.**	**Mortality percentage (%) in different fractions of rhizome extract.**		
		Methanol	**DCM**	**Hexane**
500	2.69	100	100	85.71
500	2.69	100	92.86	92.86
500	2.69	100	100	78.57
100	2	92.86	35.71	64.29
100	2	78.57	42.86	85.71
100	2	64.29	42.86	78.57
10	1	35.71	14.29	42.86
10	1	42.86	28.57	57.14
10	1	14.29	14.29	35.71
1	0	14.29	7.14	21.43
1	0	7.14	14.29	28.57
1	0	7.14	7.14	14.29
LC50 values (µg/ml)		**11.42**	**37.12**	**14.12**