“Development of curcumin-loaded silicone oil-based Janus emulsion for the management of malignant melanoma”

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By

Rhythm Bassi (Reg. No. 11507545)

Under the Guidance of

Dr. S. Tamilvanan

(Professor)

School of Pharmaceutical Sciences

Lovely Faculty of Applied Medical Sciences

Lovely Professional University

PUNJAB-144411

INDIA

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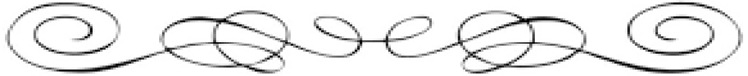
Dedicated to my parents, my mentor and God

without whom this work

could have not been possible



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Supervisor Name : Dr. S. Tamilvanan **UID :** 16391 **Designation :** Professor

Qualification : ________________________ **Research Experience :** ________________________

SR.NO.	NAME OF STUDENT	REGISTRATION NO	BATCH	SECTION	CONTACT NUMBER
1	Rhythm Bassi	11507545	2015	Y1507	8146277591

SPECIALIZATION AREA : Pharmaceutics **Supervisor Signature:** ____________________

PROPOSED TOPIC : Development of curcumin-loaded silicon oil-based Janus emulsion for the management of malignant melanoma

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PAC Member 1 Name: Dr. Amit Mittal	UID: 13145	Recommended (Y/N): NA
PAC Member 2 Name: Saurabh Singh	UID: 12208	Recommended (Y/N): NA
PAC Member 3 Name: Dr. S. Tamilvanan	UID: 16391	Recommended (Y/N): Yes
PAC Member 4 Name: Dr. Navneet Khurana	UID: 18252	Recommended (Y/N): NA
DAA Nominee Name: Dr. Sazal Patyar	UID: 17050	Recommended (Y/N): Yes

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Rhythm Bassi

Forwarded Through

Dr. S. Tamilvanan
Coordinator
Domain of Pharmaceutics
School of Pharmaceutical Sciences
LFAMS, LPU

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Place:

Date:

Dr. S. Tamilvanan
Professor
Coordinator
Domain of Pharmaceutics
School of Pharmaceutical Sciences
LFAMS, LPU

Dr. Monica Gulati
Senior Dean
School of Pharmaceutical Sciences
LFAMS, LPU

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Place: Rhythm Bassi

Date:

Abstract

The Janus emulsion containing processed and unprocessed curcumin (CUM) were developed by dispersing the curcumin in olive and silicone oils combination (1:4 ratio) and mixing the curcumin dispersed oil phase into the Tween 80 containing aqueous dispersion medium by magnetic stirrer initially followed with high speed electrical stirring. Processing of the curcumin was performed either by modified vapour pressure diffusion method and anti-solvent precipitated technique that led to the formation of curcumin pure crystal and curcumin powdered particles, respectively. Incorporating the processed curcumin appeared to enhance the drug entrapment efficiency value (from 46.83 % observed with unprocessed curcumin-loaded Janus emulsion to 75.63 - 80.03 % observed with processed curcumin-loaded Janus emulsion. It was due to the accumulation of pure crystals and powdered curcumin at the oil-in-water interface of the Janus emulsion (from 15.29 % observed with unprocessed curcumin to 24 – 47 % observed with processed curcumin). There was a formation of double-head faced structure of the dispersed oil droplets wherein the curcumin particles occupied one-head faced structure leaving the another head faced structure vacant to entrap another drug molecule with different solubility characteristics. In this context, asafoetida (botanical name: *Ferula asafoetida*, family: *Apiaceae*) was chosen to entrap inside the vacant double-head faced structure of the Janus emulsion. An improvement or significant reduction against the *in vitro* activities like antiinflammatory effect and antioxidant activity was observed for Janus emulsion containing processed curcumin in comparison with the corresponding activities observed for Janus emulsion containing unprocessed curcumin and curcumin solution or reference drug product. The presence of silicone oil in the Janus emulsion formula or the presence of curcumin drug particle at oil-water interface of the Janus emulsion was unequivocally influenced the % haemolysis values observed during RBC breakdown assay for the olive oil-based blank Janus emulsion and the olive and silicone oils-based Janus emulsion containing processed and unprocessed curcumin. Nevertheless, the beneficial effects such as anti-inflammatory and anti-oxidant activities could be of clinical interest when topically applying the olive and silicone oils-based Janus emulsion containing processed and unprocessed curcumin onto the human skin affected by skin melanoma condition.

Keywords: curcumin, Janus emulsion, silicone oil, olive oil, antiinflammatory, antioxidant, handbag structure, asafoetida.

ABBREVIATIONS

2D NOESY	:	2D Nuclear overhauser effect spectroscopy
BCC	:	Basal cell carcinoma
Bcl-2	:	B cell lymphoma 2
COA	:	Certificate of analysis
Conc.	:	Concentration
CUM	:	Curcumin
CYP1A1	:	Cytochrome P450 1A1
CYP2B1	:	Cytochrome P450 2B1
CYP2C9	:	Cytochrome P450 2C9
CYP3A4	:	Cytochrome P450 3A
DDW	:	Double Distilled Water
DEE	:	Diethyl ether
DMSO	:	Dimethyl Sulfoxide
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
DSC	:	Differential Scanning Calorimeter
EDTA	:	Ethylenediaminetetraacetic acid
eq.	:	Equation
FDA	:	Food and Drug Administration
Fig.	:	Figure
FTIR	:	Fourier transform infrared
g	:	Gram
GRAS	:	Generally recognised as safe
He-Ne	:	Helium-neon
hr	:	Hour
i.e.	:	That is
ICH	:	International Conference on Harmonization
IR	:	Infra-red
JE	:	Janus emulsion
JNPs	:	Janus nanoparticles
L	:	Litre

LOD	:	Limit of detection
LOQ	:	Limit of quantification
mg	:	Milligram
min.	:	Minute
mL	:	Millilitre
MMP	:	Matrix metalloproteinase
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NMR	:	Nuclear magnetic resonance
No.	:	Number
NPs	:	Nanoparticles
PBS	:	Phosphate buffer solution
P-gp	:	P- glycoprotein
PI3K	:	Phosphoinositide-3 kinase
PTA	:	Phosphotungstate
R^2	:	Correlation coefficient
rpm	:	Rotations per minute
S.D.	:	Standard deviation
SEM	:	Scanning electron microscope
SSMM	:	Superficial spreading melanoma
STAT-3	:	Signal transducer and activator of transcription 3
TEM	:	Transmission electron microscope
TLC	:	Thin layer chromatography
USP	:	United states pharmacopeia
UV	:	Ultra violet
λmax	:	Maximum wavelength
%	:	Percentage
µm	:	Micrometre
cP	:	Centipoise
J/g	:	Joule/gram

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Introduction

CHAPTER- 1

INTRODUCTION

1.1 Skin

Skin is the largest organ of the body, making up 16 % of the body weight, with a surface area of 1.8 m^2. The most important function of the skin is it acts as the barrier to the environment, allowing and limiting the inward and outward passage of water, electrolytes and various substances while providing protection against micro-organisms, UV radiations and toxic agents (Ramya et al., 2013).

1.1.1 Structural layers of the skin

Skin is the dynamic organ in constant state of change, as cells of the outer layers are continuously shed and replaced by inner cells moving up to the surface. Although, structurally consistent throughout the body, skin varies in thickness according to anatomical site and age of the individual (Fig. 1.1.).

The epidermis is the outer most layer, serving as the physical and chemical barrier between the interior body and interior body and exterior environment; the dermis is the deeper layer providing structural support of the skin, below which is a loose connective tissue layer, the subcutis or hypodermis which is an important depot of fat see Table 1.1.

Table 1.1. Underlying layers of skin

Skin layer	Description
Epidermis	The external layer mainly composed of the layers of keratinocytes but also containing melanocytes, Langerhans cells and Merkel cells
Basement membrane	The multilayered structure forming the dermoepidermal junction
Dermis	The area of supportive connective tissue between the epidermis and the underlying subcutis: contains sweat glands, hair roots, blood and lymph vessels

Subcutaneous	The layer of loose connective tissue and fat beneath the dermis

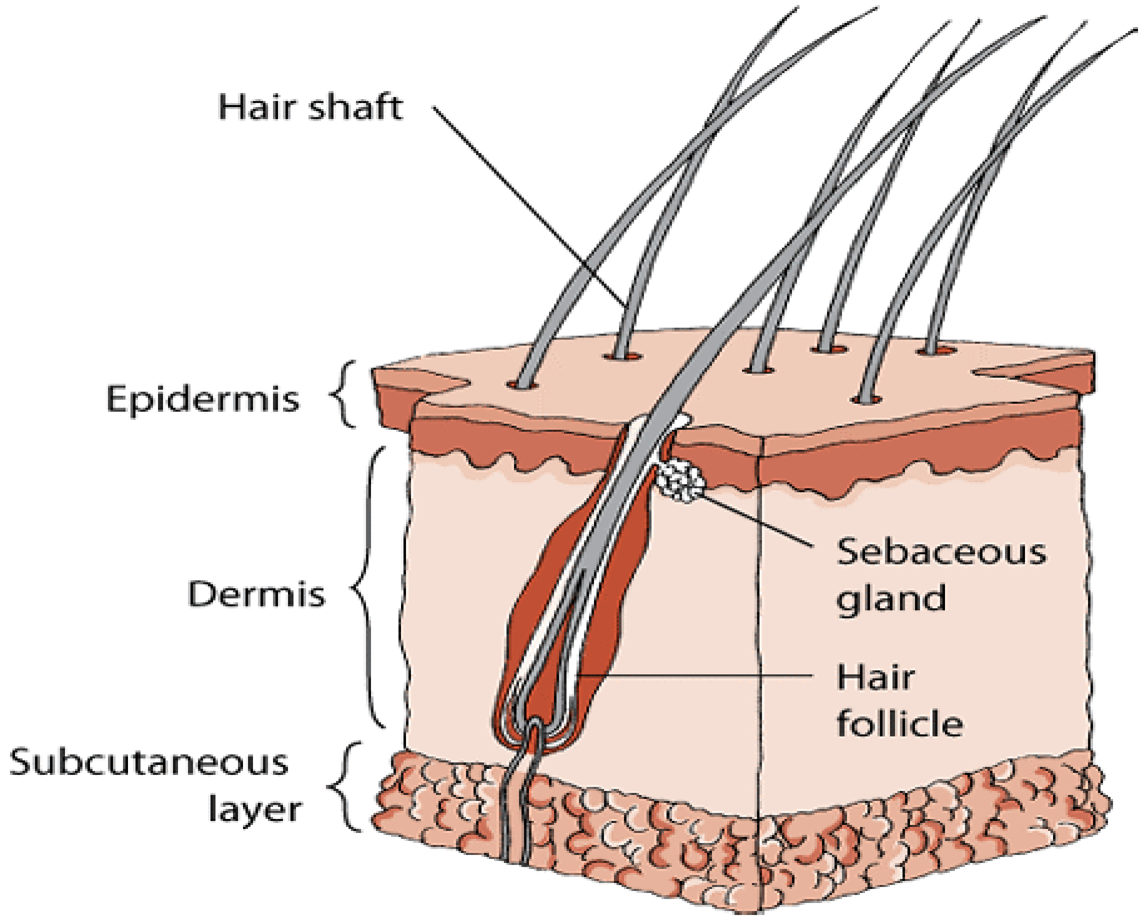


Fig. 1.1. Layers of skin

(Source: https://www.google.co.in/search?biw=1366&bih=659&tbm=isch&q=skin+layers+diagram+for+kids&sa=X&ved=0ahUKEwiz3M-Jw-DTAhWFNI8KHfazBvAQhyYILA ,accessed on 07-May-2017)

1.1.2 Cell-based division of Skin

The skin is basically divided into three types based on the type of cells as in Fig. 1.2:

(1) Squamous cells

These are *flat cells* in the outer part of the epidermis that are constantly shed as new ones form.

(2) Basal cells

These cells are in the lower part of the epidermis, called the *basal cell layer*. These cells constantly divide to form new cells to replace the squamous cells that wear off the skin's surface. As these cells reach the surface, they get flatter and eventually become squamous cells.

(3) Melanocytes

These are the cells that can become melanoma. They make a brown pigment called *melanin*, which gives the skin its tan or brown color. Melanin protects the deeper layers of the skin from some of the harmful effects of the sun. When the skin is exposed to sunlight, Melanocytes make more of the pigment, causing the skin to tan or darken.

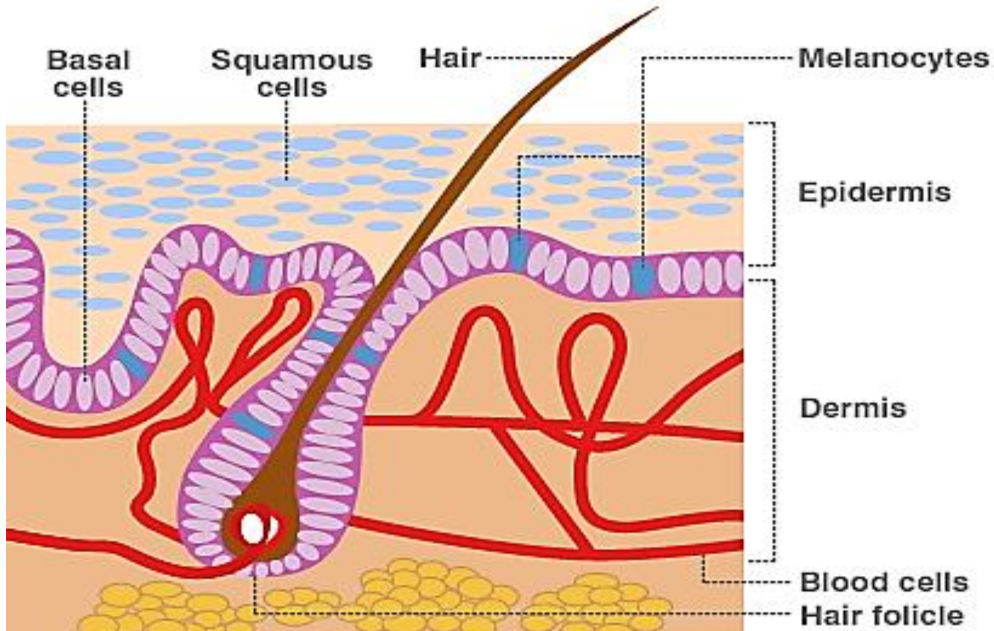


Fig 1.2: Cells present on the skin surface

(Source: https://www.google.co.in/search?biw=1366&bih=659&tbm=isch&q=skin+cells+diagram&sa=X&ved=0ahUKEwiakdjow-DTAhVCNo8KHQI_AccQhyYILA, accessed on 07-May-2017)

1.2 Skin carcinoma

Skin carcinoma is defined as the abnormal growth of cells that lead to the development of cancer. Skin Carcinoma starts when the cells in skin layer begin to grow out of control. Skin cancer can be classified as melanoma and non-melanoma type of cancer, depending on the type of cells involved. Malignant melanoma is the most serious type of skin cancer. Others, non-melanoma is not more common though are less serious, but still needs treatment. Skin cancer can start anywhere, even in places that are rarely exposed to the sun. Melanoma can spread inside the body too. It appears as a new mole or mark. Unlike modes, skin cancer cells do spread and break away from the original growth and enter the blood vessels or lymph vessels. The cancer cells may found in lymph nodes and spread to other tissues attached there to form new tumors that may damage those tissues (US department of health and human services).

1.2.1 Key statistics for skin cancer

The American Cancer Society's estimates for melanoma in the United States for 2016 are:

- About 76,380 new melanomas will be diagnosed (about 46,870 in men and 29,510 in women).
- About 10,130 people are expected to die of melanoma (about 6,750 men and 3,380 women).

The rates of melanoma have been rising for the last 30 years (American Cancer Society).

1.2.2 Risk of Developing Melanoma

Melanoma is more than 20 times more common in whites than in African Americans. Overall, the lifetime risk of getting melanoma is about 2.5% (1 in 40) for whites, 0.1% (1 in 1,000) for blacks, and 0.5% (1 in 200) for Hispanics. The risk of melanoma increases as people age. The average age of people when it is diagnosed is 63. But melanoma is not uncommon even among those younger than 30. In fact, it's one of the most common cancers in young adults especially young women (American Academy of Pediatrics).

1.2.3 Ultraviolet (UV) light exposure

UV rays exposure is the major risk factor for most of Melanoma. The major source of UV rays is sunlight; others include tanning beds and sun lamps. UV rays damage the

DNA of skin. The skin cancer begins when this damage affects the DNA of gene that control skin cell growth. DNA is the chemical in each of our cells that makes up our genes, which control how our cells function. We usually look like our parents because they are the source of our DNA. But DNA affects more than just how we look. Some genes control when our cells grow, divide into new cells, and die:

- Genes that help cells grow, divide, and stay alive are called ***oncogenes***
- Genes that keep cell growth in check or cause cells to die at the right time are called ***tumor suppressor genes***

Cancers can be caused by DNA changes that turn on oncogenes or turn off tumor suppressor genes. Changes in several different genes are usually needed for a cell to become a cancer cell. Ultraviolet (UV) rays are clearly a major cause of melanoma. UV rays can damage the DNA in skin cells. Sometimes this damage affects certain genes that control how skin cells grow and divide (Ghissassi et al., 2009). If these genes no longer work properly, the affected cells may become cancer cells.

Keys note to be followed to lower the risk of melanoma cancer:

- Limit exposure to UV rays
- Avoid using tanning beds and sunlamps
- Watch abnormal moles
- Avoid weakening your immune response

1.2.4 Types of Skin Cancer

Based on the location of area where the cancer development is seen, the cancer is classified as:

1.2.4.1 Basal Cell Carcinoma (BCC)

The most common type of the skin cancer that usually occurs in head and neck regions followed by trunk and extremities regions. The cancer arises in the basal layer of epidermis (Chummun et al., 2011). The BCC is further classified into **three types**: Superficial, Nodular and Sclerosing.

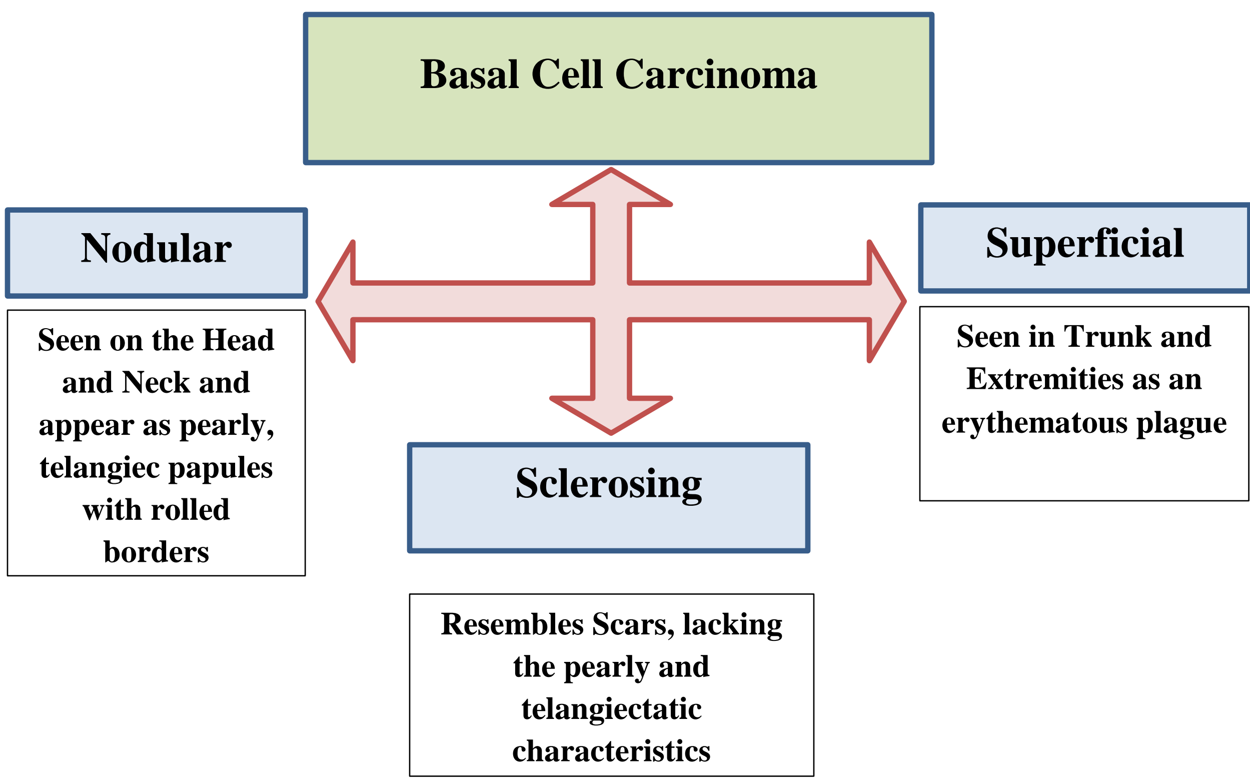


Fig. 1.3. Classification of Basal Cell Carcinoma

1.2.4.2 Melanoma

This is less common than other skin cancers and is the most dangerous of all causing majority (75 %) of deaths. Generally associated with melanocytes of epidermal layer, that synthesizes melanin pigment that produces cells of skin with photo protection from mutagenic UV-rays. Melanoma Cancer is the cancer that usually starts in Melanocytes cells. These are also known as Malignant Melanoma and Cutaneous Melanoma. During the cancer development, most melanoma cells still make melanin, so such cancer appears black or brown. But some melanomas don’t make melanin and appear pink, tan or white. Melanoma cancer can develop anywhere on skin and are more likely to start on trunk (Chest and back) in men and legs in women. Face and neck are other common sites. Such cancer is less common than basal and squamous type but is more dangerous. Malignant melanoma involves the formation of mole (nevus) that is benign skin tumor that develops from Melanocytes. Melanoma accounts for only about

1% of skin cancers but causes a large majority of skin cancer deaths (Langley et al., 2003).

1.2.4.3 Squamous Cell Carcinoma

This is considered as second most common type of cancer and has the potential of life-threatening situation that occurs on sun exposed sites of head and neck. It is generally seen in elderly people due to weak immune system power. Immunosuppressive drugs like cyclosporine, steroids and azathioprine can cause the increase incidence of skin cancer (Gianni et al., 2005).

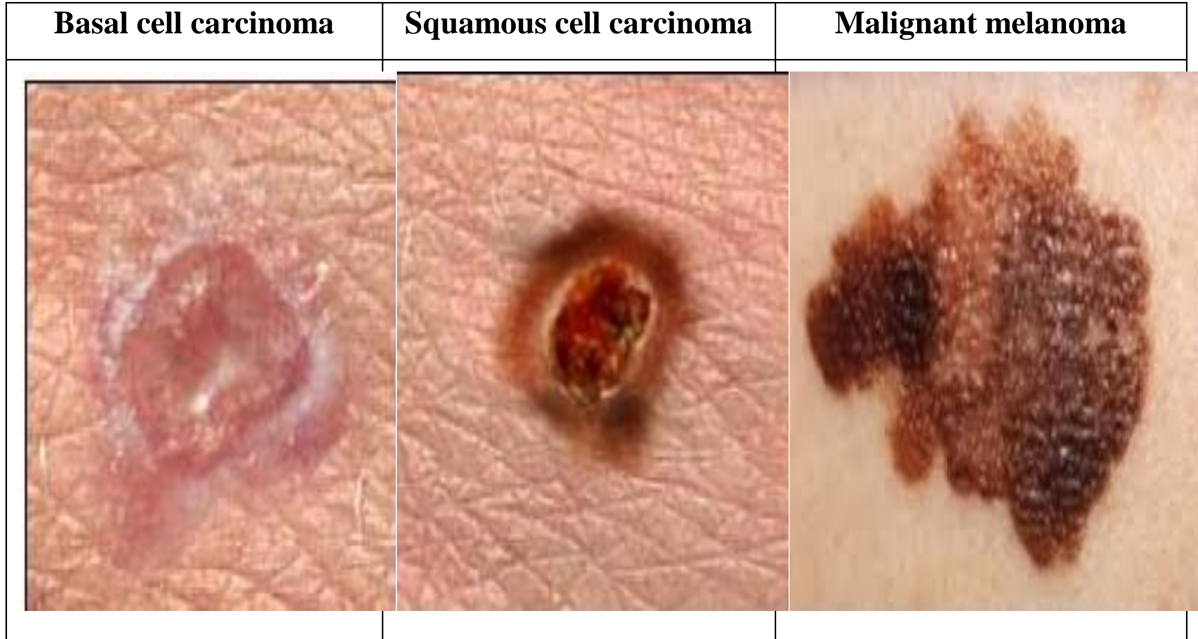
Basal cell carcinoma	Squamous cell carcinoma	Malignant melanoma
		

Fig. 1.4. Morphology of three different type of skin cancer

(Source: Silpa et al., A review on Skin cancer. Int. Res. J. Pharm.2013, 4(8))

1.2.5 Treatment of Skin Carcinoma

The choice of the treatment depends onto the histological type of the lesion, its size and location, more importantly on the age of the patient. However no such specific treatment is being assigned in the treatment of the skin cancer, the best way is the prevention of UV light exposure. The treatment goals can be the total removal of the tumor, preservation of function, and a good cosmetic outcome.

1.2.6 Severity of Skin cancer

- ✓ 80 % Basal Cell Carcinoma
- ✓ 16 % Squamous Cell Carcinoma
- ✓ 4 % Melanoma

1.2.7 Malignant melanoma

Malignant melanoma (MM) is the malignancy of pigment-producing cells (melanocytes), which are located primarily in the skin, but also found in the ears, gastrointestinal tract, eyes, oral and genital mucosa and leptomeninges. It has the highest majority rate with more than 2000 deaths UK wide in 2011 (Cancer Research UK, 2014). In Northern Ireland, the numbers of melanoma cases have increased from 103 cases per year in 1984-1992, to 258 per year in 2004-2009 (Hunter et al., 2013).

1.2.7.1 Pathophysiology of malignant melanoma

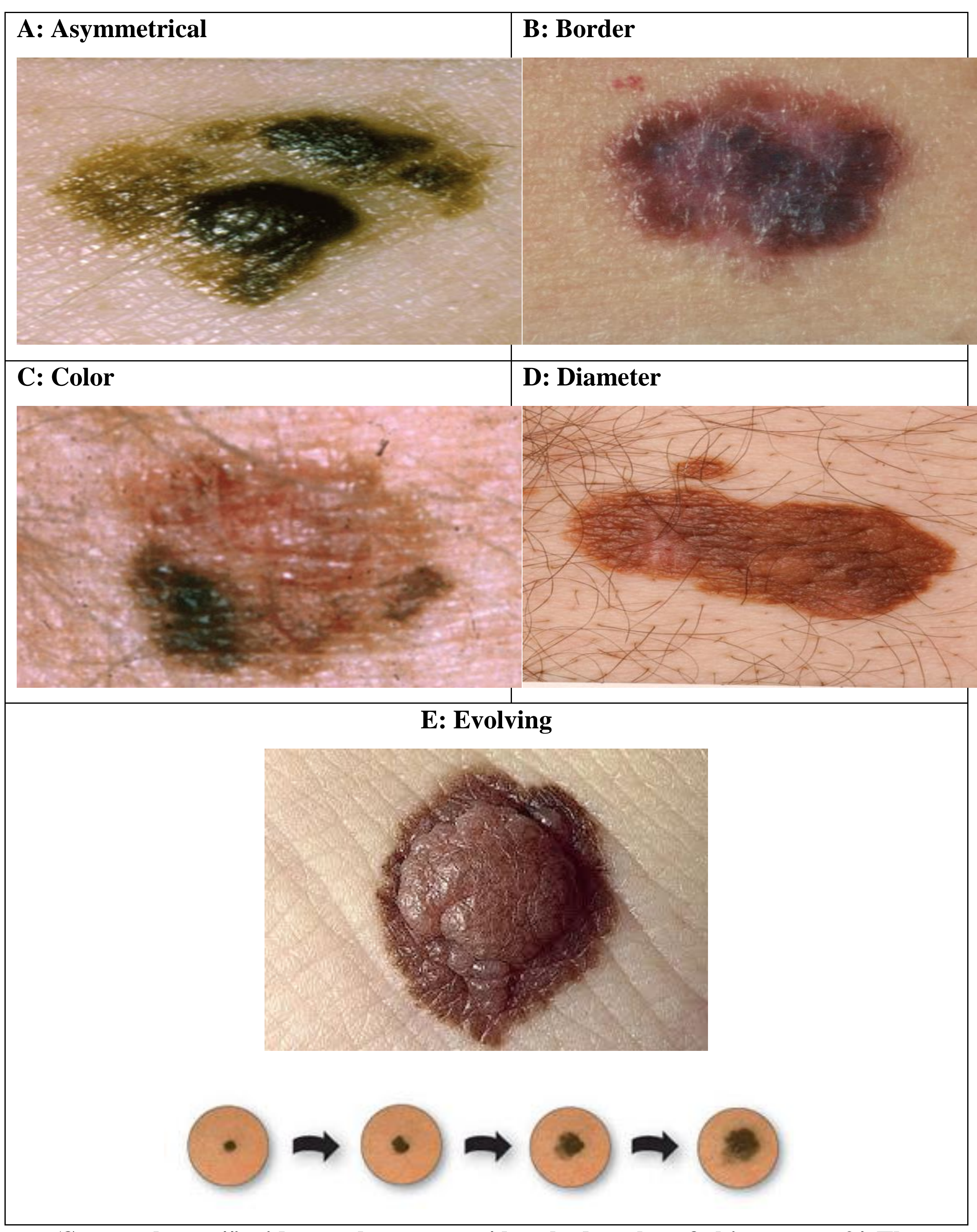
The sequence of events whereby normal melanocytes transform into melanoma cells (melanogenesis). This is mainly due to multistep process of genetic mutations that alter the cell cycle and render the melanocytes more susceptible to the carcinogenic effects of UV radiations (Demierre et al., 2003).

1.2.7.2 Classification of malignant melanoma

The melanoma can be classified into four different clinical subtypes:

- Superficial spreading melanoma (SSMM)
- Lentigo maligna melanoma
- Nodular melanoma
- Acral lentiginous melanoma

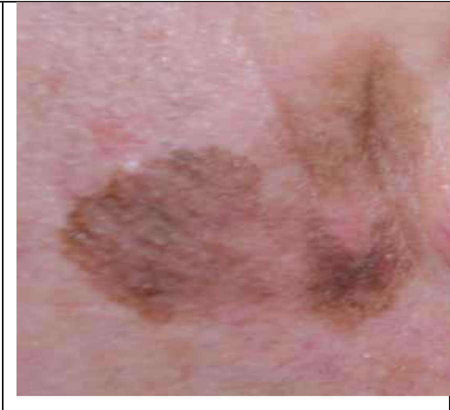
Table 1.2. ABCDE of melanoma

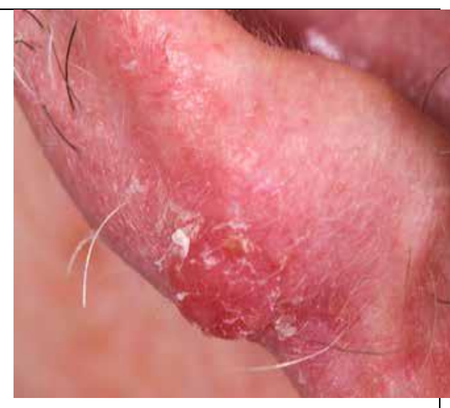
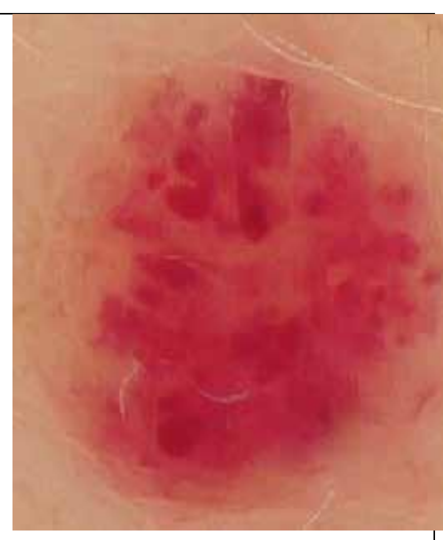


(Source: https://insideouterbeauty.com/the-abcde-rules-of-skin-cancer-2/, The American academy of Dermatology, accessed on 07-May-2017)

Table 1.3. Features of the individual cancer based on ABCDE rule

<table>
<tr><td rowspan="6">Superficial spreading melanoma (SSMM)</td><td>Tend to present as a flat or slightly elevated brown lesion with variegated pigmentation (i.e. black, blue, pink or white discoloration) with an irregular shape often > 6 mm</td><td colspan="2">ABCDEs of melanoma</td></tr>
<tr><td rowspan="5"></td><td>A</td><td>Asymmetry</td></tr>
<tr><td>B</td><td>Border irregularity</td></tr>
<tr><td>C</td><td>Color variation</td></tr>
<tr><td>D</td><td>Diameter > 6 mm</td></tr>
<tr><td>E</td><td>Evolving (changing)</td></tr>
</table>

Lentigo Maligna melanoma	Present as the slow growing or changing patch of discolored skin with variegated shape and color. Detected using ABCDE rule.	

Nodular melanoma	These are may be non-pigmented and may be ulcerated.	
Acral lentiginous melanoma	Starts as a slowly enlarging flat patch of discolored skin and tends to follow the ABCDE rule. Initially, smooth surface, later becomes thicker and irregular surface.	

1.3 Curcumin (CUM) : The spice for life

1.3.1 Biological Source of CUM

Curcumin (CUM) is the principal curcuminoid of the popular Indian spices turmeric, derived from the rhizomes of *Curcuma longa*, member of the ginger family (*Zingiberaceae*). In the Ayurvedic tradition, turmeric or haldi as it is known in Hindi with its main action being to reduce mucus from the system. The other two curcuminoids present are demethoxycurcumin and bisdemethoxycurcumin. The curcuminoids are polyphenols and are basically responsible for the yellow color of the turmeric (Akram et al., 2010)**.**

1.3.2 Chemical structure of CUM

CUM can exist in at least two tautomeric forms, keto and enol. Under which enol form is more energetically stable in solid phase or solution. CUM is brightly yellow colored and used in food coloring. It easily reacts with the boric acid forming red colored compound known as rosocyanine.

Fig. 1.5. Chemical structure of CUM

According to the structure (Fig. 1.5) it is concluded that the methoxy groups on the phenyl rings in CUM are important to have health effects (Sandur et al., 2007).

Besides CUM, there are others components in *Curcuma longa* called the CUMoids group- demethoxycurcumin and bisdemethoxycurcumin. CUM is found to be abundant of all (nearly 70 % of the total).

1.3.2.1 Major chemical constituents of turmeric

The extract of turmeric contains following constituents based on Fig 1.6.

- 70% CUM
- 17% Demethoxycurcumin
- 3% Bisdemethoxycurcumin

H3CO OCH3 HO OH Curcumin I (≈70%)

HO OCH3 OH Demethoxycurcumin Curcumin II (≈17%)

HO OH Bis-Demethoxycurcumin Curcumin III (≈3%)

Fig. 1.6. Other components present in turmeric

1.3.3 Structural characteristic of CUM

CUM is the synthetic molecule, also known as diferuloyl methane. The diketo group present exhibits keto-enol tautomerism, which further exit in different types of conformers depending on the environment (Priyadarsini, 2009).

In the crystal state it exists in a *cis*-enol, where it is stabilized by resonance assisted hydrogen bonding. The dipole moment of CUM in the ground state is 10.77 D.

1.3.4 Availability of CUM

Curcuma longa has been traditionally used in Asian Countries, China and Indonesia as a medical herb for several pathologies. It is a common spice, known mostly for its use in Indian dishes as a common ingredient in curries and other ethic meals. Turmeric has also been used from centuries in Ayurvedic medicine, which integrates the medical properties of herb with food. The extraordinary herb found its way into spotlight in the west due to its medical benefits. Traditionally, turmeric has been used topically to heal and reduce bleeding associated with bruises, spains, leech bites and inflamed joints. It has also been used internally for liver and digestive complaints, menstrual insufficiency and in jaundice treatment.

1.3.5 Therapeutic activities of CUM with its mechanism of action

1.3.5.1 Antioxidant effect

Water- and fat- soluble extracts of turmeric and its CUM component exhibit the strong antioxidant activity compared to the Vitamin C and E. CUM was shown to be eight times more potent than the vitamin E in lipid peroxidation and three times more powerful than vitamin C in neutralizing free radical. The anti-oxidant effect of the CUM is mediated through antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase. CUM has shown to serve as a Michael acceptor, reacting with glutathione and thioredoxin.

1.3.5.2 Hepatoprotective effects

Hepatoprotective effects are mainly due to CUM's ability to decrease the formation of pro-inflammatory cytokines. Sodium curcuminate, a salt of CUM, also exerts choleretic effects on increasing biliary excretion of bile salts, cholesterol as well as increasing the bile solubility, therefore possibly preventing and treating cholelitiasis.

1.3.5.3 Antiinflammatory effects

The antiinflammatory property maybe attributed to its ability to inhibit both biosynthesis of inflammatory prostaglandins from the arachidonic acid and neutrophil function during inflammatory states. CUM can also be applied topically to counteract inflammation and irritation associated with inflammatory skin conditions and allergies.

1.3.5.4 Anticarcinogenic effect

In vitro studies utilizing human cell lines have demonstrated CUM's ability to inhibit carcinogenesis at three stages: tumor promotion, angiogenesis and tumor growth. CUM is capable of suppressing the activity of several mutagens and carcinogens in variety of cell types. The anticarcinogenic effect is due to the direct antioxidant and free radical scavenging effects as well as ability to indirectly increase glutathione levels and inhibiting nitrosamine formation.

1.3.5.5 Antimicrobial effect

CUM and the various essential oils present in the turmeric inhibit the growth of variety of bacteria, parasites and pathogenic fungi. A study on chicks infected with the caecal parasite *Eimera maxima* demonstrated that the diet supplemented with 1 % of CUM resulted in the small intestinal lesion scores and improved weight gain.

1.3.5.6 Cardiovascular effects

CUM shows its action via lowering the cholesterol and triglyceride levels and inhibiting platelets aggregation. Such effects are noted even at low concentration of CUM. This is due to the decrease in the cholesterol uptake in the intestines and increased conversion of cholesterol to bile acids in the liver. Furthermore, the inhibition of platelet aggregation by *C. longa* constituents is thought to be *via* potentiation of prostacyclin synthesis and inhibition of thromboxane synthesis.

1.3.5.7 Gastrointestinal effects

CUM shows the protective effects on the gastrointestinal tract. Sodium curcuminate inhibited intestinal spasm and p-tolymethylcarbinol, a turmeric component, increased gastrin, secretin and pancreatic enzyme secretion. Turmeric also inhibits the ulcer formation caused by stress, alcohol and significantly increasing the gastric wall mucus.

1.3.5.8 CUM enhances immunity

CUM helps the body to fight off cancer should some cells escape apoptosis. When researchers looked at the lining of the intestine after the ingestion of the CUM, they found that CD4+ T-helper and B type immune cells were greater in number in comparison to the localized immune stimulation. It is well documented that after the ingestion of CUM there is increase in antibodies and hence, greater immune response.

1.3.5.9 Pregnancy and lactation

There is no evidence that dietary consumption of turmeric as a spice adversely affects pregnancy or lactation, the safety of CUM supplements in pregnancy has not been established.

1.3.6 Bioavailability and metabolism of CUM

In order to determine the biological availability of CUM, Wahlstrom and Blennow in 1978, where CUM was administered to Sprague –Dawley rats at a 1 g/kg dose. In this study a low level of CUM was observed in blood plasma of rats (Wahlstrom et al., 1978). However, latter studies demonstrated that when CUM was administered orally at the dose of 2000 mg/kg to rats; the maximum serum concentration was 1.35 ± 0.23 μg/mL although in humans it was undetectable (Shoba et al., 1998).

The oral bioavailability of CUM in rats was around 1 %, so very high doses of CUM are necessary (3600 to 12,000 milligrams) to achieve its beneficial effects (Yang et al., 2007). In the latter study it has been shown that CUM metabolites were detected in plasma when patients ingested at least 3600 milligrams of CUM being mainly detected as CUM glucuronides and CUM sulphate. In urine samples, CUM and its metabolites were detected primarily as CUM, flowed by glucuronides and finally in the suphates forms.

1.3.7 Pharmacokinetic properties of CUM

The pharmacokinetic studies in animals have demonstrated the 40-85 % of the oral dose of CUM passes through the gastrointestinal tract unchanged, with most of the absorbed flavonoids being metabolized in the intestinal mucosa and liver (Wahlstrom et al., 1978; Ravindranath et al., 1980).

1.3.8 Water solubility of CUM

CUM is the hydrophobic molecule with log P value of 3.0. It is almost insoluble in water and readily soluble in polar solvents like DMSO, methanol, ethanol, acetonitrile, chloroform, ethyl acetate, etc. it is sparingly soluble in hydrocarbons solvents like cyclohexane and hexane. CUM possesses less than 0.1 mg/ml water solubility.

The aqueous CUM solutions can be prepared by adding surfactants, lipids, albumins, cyclodextrins, biopolymers etc. to form a micelle solution. Micellar solutions using surfactants are the best suited for preparing high concentration CUM solutions in water (Priyadarsini, 2009).

1.3.9 CUM as anticarcinogenic

The in-vitro studies utilizing human cell lines, have demonstrated the CUM's ability to inhibit Carcinogenesis at three stages that are **tumor promotion**, **angiogenesis** and **tumor growth** (Aggarwal et al., 2007).In two studies of colon and prostate cancer, CUM shows its effect by inhibiting cell proliferation and tumor growth. CUM is capable of suppressing the activity of several mutagens and carcinogens in variety of cells. The carcinogenic effect due to **antioxidant and free radical scavenging effects**, ability to indirectly increase Glutathione levels; there by aiding in hepatic detoxification of mutagens and carcinogens and inhibiting nitrosamine formation (Goel et al., 2008).

CUM appears as a promising chemo preventive compound able to reverse, inhibit or prevent the development of cancer by inhibiting specific molecular signaling pathways involved in carcinogenesis (Hatcher et al., 2008; Thangapazham et al., 2006).

Furthermore, carcinogenesis is the multistage process with three successive steps, initiation, promotion and progression. This process is also linked to oxidative stress, chronic inflammation and hormonal imbalance. The Chemo preventive effect of CUM is based upon its effectiveness to inhibit tumor genesis through the decrease of cancer cell proliferation.

1.3.9.1 Mechanism of cell Death induced by CUM

CUM causes the tumor cell death through the induction of apoptosis process. The major drawback with the apoptosis process is the tumor cell resistance that appears frequently and associated with poor prognosis and resistance to cancer treatment (Bruin et al., 2008).

CUM also induces other types of mechanism leading to cell death that are autophagy, mitotic catastrophe) in order to compensate the previously explained lack of induction of cell death mechanisms with reference to Fig. 1.7.

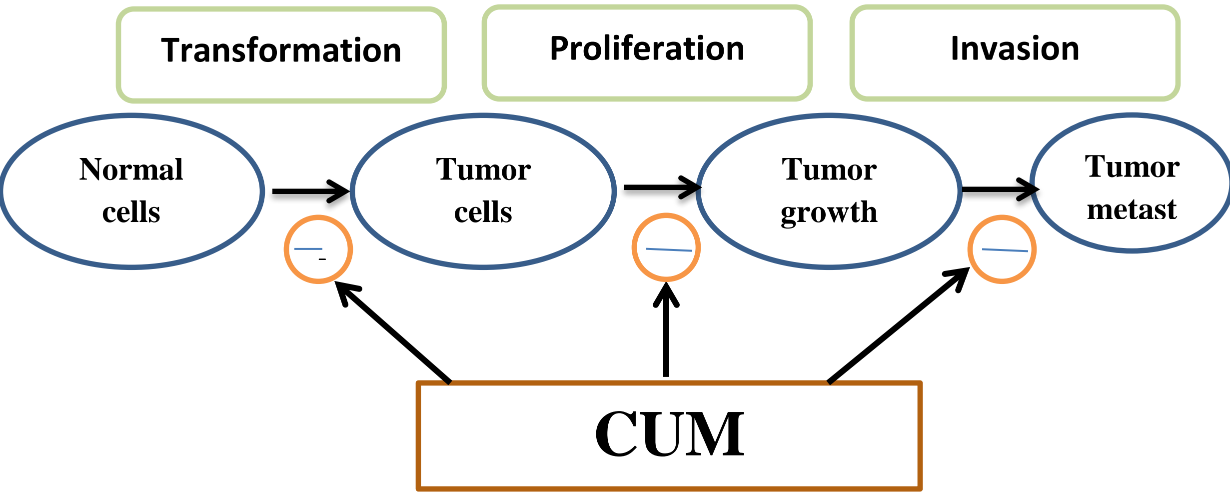


Fig. 1.7. Action of CUM on cancer cells

1.3.10 Complications with CUM

CUM is hampered by its poor solubility and short biological half-life, resulting in low micromolar range bioavailability in both plasma and tissues. In order to overcome this limitation, several methodologies have been tested *in-vitro* that include its combination with various adjuvants (e.g. piperine), and the development of delivery vehicles with incorporation of liposomes, nanoparticles and phospholipid formulation of CUM (Cheng et al., 2001; Anand et al., 2007; Burgor et al., 2009).

1.3.11 Clinical indications of CUM

The CUM shows abundant position in its clinical indications that are as followed:

- Generalized chronic inflammation
- Uveitis

- Chemoprevention with specific indication to resuce the risk of colorectal and pancreatic cancers as well as multiple myeloma
- Age-related cardiovascular disease
- Osteoarthritis
- Dyspepsia, irritable bowel syndrome and gastric ulceration

1.3.11.1 Dosage range

Doses of 500-8,000 mg of powdered turmeric per day have been used in human studies. The standardized extracts are typically used in lower amounts, in the 250-2,000 mg range.

1.3.11.2 Contraindications

CUM have been granted as “Generally Recognized as safe” (GRAS) status in the United States of America by the Food and Drug Administration (FDA), turmeric is well tolerated by most of the people. CUM extract does not up regulate P-gp (Hou et al., 2008). CUM inhibits CYP3A4 and CYP2C9 and is itself metabolized by CYP1A1 and CYP2B1. Therefore, there is the theoretical possibility of interference with the drug metabolized through CYP3A4. However, it is possible that CUM lacks clinically significant (in vivo) CYP3A4 activity (Volak et al., 2008).

1.3.11.3 Toxicity

No significant toxicity has been reported following short or long-term administration of turmeric extracts at standard doses.

1.4 Janus particles

Janus particles are the simplest case of non-centrosymmetrical particles. These particles are known after the Roman god Janus, who is represented by double-faced head. In the same manner, Janus particles have two faces with different physical and chemical properties. Several recent reviews have done an excellent job that summarizes the synthesis method and properties of Janus particles. Janus particles are microscopic particles in dimension ranging between 1-100 nm with two different sides. It is an analogue to the surfactant molecule, which has a hydrophilic head group and

hydrophobic tail. Due to its fundamental as well as commercial application, Janus partials have collectively considerable attention in drug delivery system and advanced surface-active properties with a uniform wettability. Binks and Flechter (2003) investigated the wettability of Janus beads at the interface between oil and water and conclude that Janus particles are both surface-active and amphiphilic and are more stable compared to homogeneous particles that are only surface-active.

Particle stabilized emulsion are named after Pickering as “Pickering emulsion” for his first study on particle –stabilized emulsion. The techniques used to identify JNPs, various microscopic and macroscopic methods are used. Microscopic methods include electron microscopy and scanning probe microscopy that give a very accurate information about the morphology but not useful to quantify the polydisperse population of morphologies, i.e., cannot differentiate between relative sizes of patches of JNPs and NPs and whereas, Macroscopic methods include 2D NOESY NMR, contact angle that measure the properties attributed to JNPs but they often cannot distinguish between JNPs and NPs.

1.5 Janus Emulsion

Emulsion is referred to as dispersion of droplets of one fluid within second immiscible fluid. These involve shearing of two immiscible fluids, leading to the fragmentation of one phase into another phase. These types of emulsions are intrinsically unstable because of large interfacial area within the system. To obtain stable emulsion, it involve the incorporation of another third component that is the emulsifying agent such as surfactant and colloidal particles that act onto the interface between the two immiscible fluids and further, stabilize the emulsion (Gangwal et al., 2008). Such emulsions have wide range of activity from food stuffs to pharmaceutical to personal care products to agro cultures. Surfactants that are used in emulsion are commonly referred to as surface active agents that are used to segregate at the interface and stabilize the emulsion. Whereas the colloidal particles have property to attach strongly to the interface in order to stabilize the emulsion. The surface activity of the surfactants is originated from their amphiphilic structure. It has been demonstrated that Janus particles too have amphiphilic character that are used in order to stabilize the emulsion thermodynamically (Walther et al., 2013).

1.5.1 Advantages of using Janus particles

The design and synthesis of Janus nanoparticles has attracted great scientific interest. Janus particles are the particles with 1 to 100 nm. The key advantage Janus particles have two different sides that are in particular size range. Hence the two parts possess different nanoscale properties (Binks et al., 2001).

Another advantage of Janus particles is that it shows high surface-to-volume ratio and further, yielding enhanced catalytic properties and strong interaction with biological samples (Crossley et al., 2010). Janus particles involve a self-assembly processes that has the ability to stabilize the interfaces. This basically shows the Pickering effect with the surfactant. These are more strongly adsorbed on the interface compared to homogenous nanoparticles. Likewise, the adsorption energy depends on the size of the particles involved. The Janus character of the Janus particle can enable the small particles to go to the interface and stabilize the emulsion.

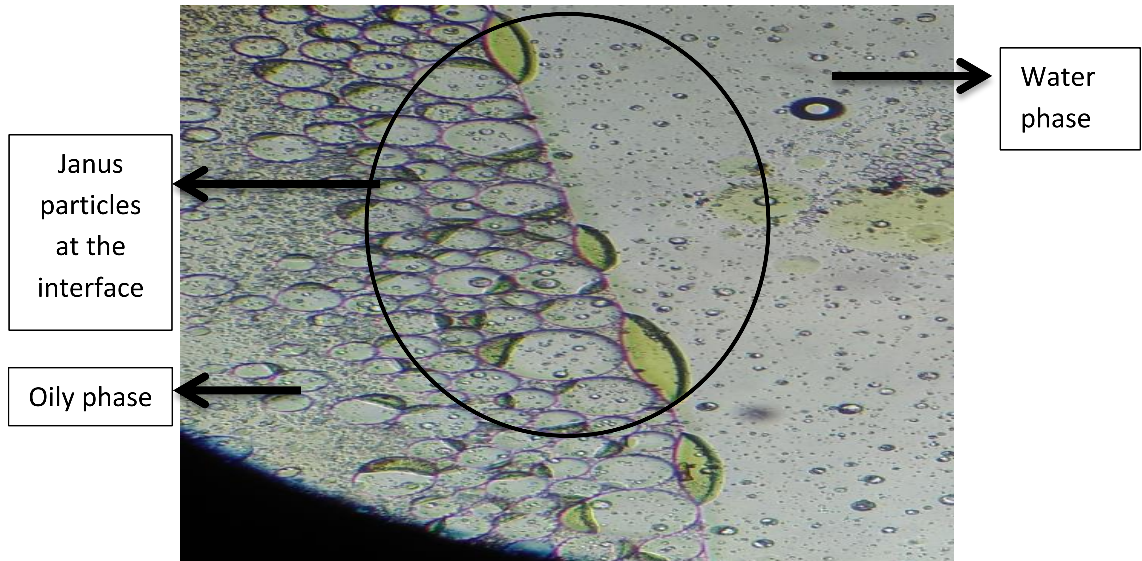


Fig 1.8. Janus particle at oil/water interface

Janus particles usually inhibit coalescence of the dispersed phase on Pickering emulsion. The two faces of the Janus particles situated at different liquid interfaces can be used for various purposes due to difference in their optical, electrical or magnetic properties (Cole-Hamilton, 2010). Taking about its bioapplications, Janus particles can easily combine with different functionalities such as targeted drug delivery system,

bimolecular bio sensing or molecular imaging. It can be illustrated by simple example of transport capabilities of protein cages with streptavidin (Suci et al., 2009) It can also be modified as using one side of the Janus particle dedicated to target specific receptors while others in sensing events (Wu et al., 2010). The high surface to volume ratio of Janus particles make it suitable for various catalytic properties (Zhang et al., 2016; Pradhan et al., 2009).

Terminology

CHAPTER -2

TERMINOLOGY

2.1 Calibration Curve

In the analytical chemistry, a calibration curve, also known as standard calibration curve, is a general method for determining the concentration of the substance in an unknown sample by comparing the unknown to a set of standard samples of the known concentration.

2.2 Handbag structures

Hand-bag structure basically represents the bi-compartment structure of dispersed oil droplets of JE. It has two vacant chambers where drug loading can be performed based upon the solubility characteristics of the drug.

2.3 Haemolysis

Hemolysis is basically defined as the rupturing or lysis of red blood cells (erythrocytes) and the release of their contents (cytoplasm) into the surrounding fluids (blood plasma). Haemolysis may occur *in vivo* and *in vitro*. It damages the host cytoplasmic membrane, causing cell lysis and death.

2.4 Janus

Janus word is derived from the 'name of the Roman god'. In the ancient Roman religion and myth, Janus is the god of beginnings, gates, transitions, time, duality, doorways, passing and endings. He is usually depicted as having two faces, since is look to the future and to the past. Janus also presided over the beginning and ending of conflict and hence war and peace.

2.5 Melanoma

Melanoma, also known as malignant melanoma, is a type of cancer that develops from the pigment-containing cells known as melanocytes. Melanoma usually occurs in

the skin but more really in the mouth, intestine or eye. They develop from a mole with concerning changes including an increase in size, irregular edges, and change in color, itchiness or skin breakdown.

2.6 Antioxidation

Antioxidant is the molecule that inhibits the oxidation of the other molecules. Oxidation is the chemical reaction that can produce free radicals, leading to the chain reactions that may damage cells. Antioxidant dietary supplements do not improve health nor they are effective in preventing diseases but useful in case of oxidation stress that is considered to cause some diseases.

2.7 Physiochemical properties

The physicochemical properties of the compound such as solubility, stability, solid-state properties, partition coefficient and ionization constants that are essential so that formulation process can be rational and streamlined. It is one of the key challenges to develop the pharmaceutical active ingredient into the drug, which combines biological activity with an appropriate physiochemical profile.

2.8 Pickering effect

The effect involved in the stabilization of the emulsion. Here, the solid particles adsorb onto the interface between the two phases. This type of emulsion is known as Pickering emulsion. The type of the emulsion named after S.U. Pickering, who described the phenomenon in 1907, although the effect was firstly recognized by Walter (1903).

2.9 Preformulation studies

The preformulation testing is the first step in the rational development of the dosage forms and mainly focus on the physiochemical properties of the new drug candidate. It is basically the physical and chemical property of the drug substance alone or combined with the excipients. The only key point of such studies is to improvise the drug performance and effective development of the dosage form.

2.10 Scavenging

Scavenging is basically a chemical substance (like DPPH) that is added into the mixture in order to remove or de-activate impurities and unwanted-reaction products (e.g. oxygen) to make that that they may not cause any unfavorable reactions.

Literature Review

CHAPTER-3

LITERATURE REVIEW

3.1 Outline on CUM

CUM, when formulated shows poor solubility, low absorption from the gut, rapid metabolism and rapid systemic elimination which in turn limits the potential therapeutic health benefits. When given orally, the foremost portion of the ingested CUM is excreted through the faeces in an un-metabolized form and the small portion that is absorbed converted to water soluble metabolites, glucuronides and sulphates. Additionally, CUM undergoes the microbial metabolism which in turn yields di-hydrocurcumin and tetra hydrocurcumin via NADPH dependent reduction.

In order to improvise the health benefits of CUM, formulation such as Janus emulsion could be proven as interesting to see the absorption, metabolism, bioavailability and the stability of Janus particle in macroemulsion. The double-head Janus particle will show its adsorption at oil-water interface which further improves the wettability of the prepared formulation. The current proposed Janus emulsion containing CUM for skin carcinoma in topical formulation and the processing of the CUM expected the following advantages:

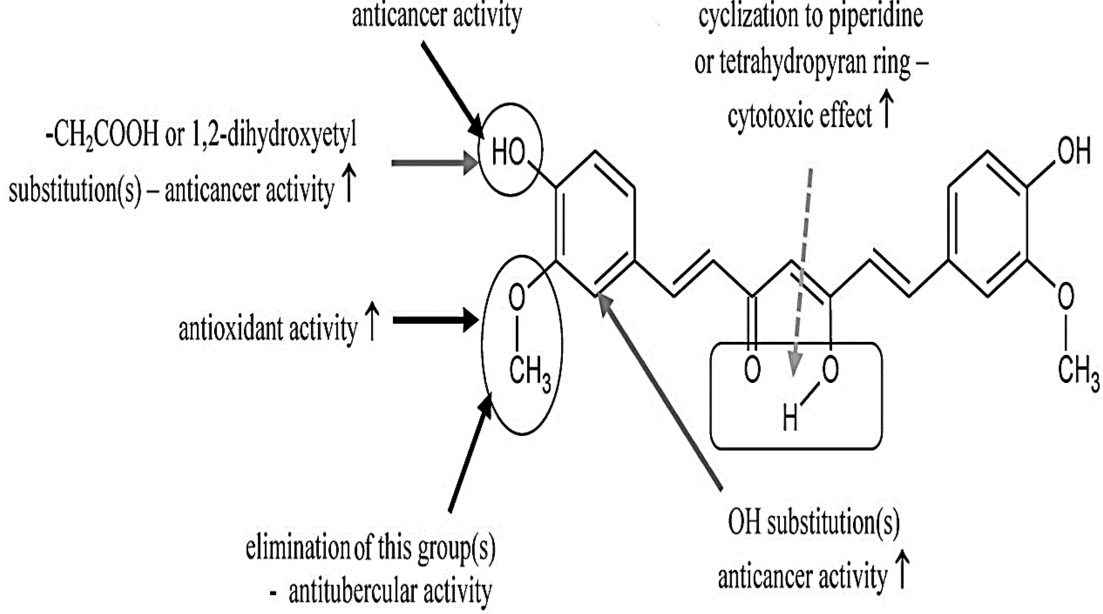
- A improved bioavailability of the CUM from Janus emulsion
- To upgrade the stability of the Janus particle in the emulsion containing minimum amount of emulsifier
- Enhanced % drug entrapment efficiency of the processed CUM and drug amount at oil-water interface of Janus emulsion

3.2 Drug profile

Drug name	Curcumin (CUM)
Chemical structure	
Molecular weight	368.38 g/mol
Chemical formula	$C_{21}H_{20}O_6$
IUPAC name	1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione
Appearance	Dark orange powder
Pharmacology	Common anti-melanoma effects of natural compounds include potentiating apoptosis, inhibiting cell proliferation and inhibiting metastasis. Mechanism include such as promotion of caspase activity, inhibition of angiogenesis and inhibition of the effects of tumor promoting proteins such as PI3-K, Bcl-2, STAT3 and MMPs (Jiang et al., 1996; Yan et al., 2005; Kuo et al., 1996)
Pharmacodynamics	Topical application of 3 or 10 µmol CUM has shown to inhibit chemical carcinogensis. Likewise, combination of CUM with other chemoprotective agents also shown augmented growth inhibitory effects (Conney et al., 2003)
Melting point	183 ° C (361 ° F; 456 K)
Class	Curcuminoids of Turmeric

Therapeutic indication	Daily consumption of CUM over a 3-month period of up to 12 grams was found to be safe in humans
Solubility	CUM is insoluble in water, soluble in polar solvents like DMSO, methanol, ethanol, acetonitrile, chloroform, and ethyl acetate and sparingly soluble in hydrocarbon solvents like chloroform and hexane

Fig. 2.1. Functional groups present on CUM with its biological action



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3.3 Literature review on CUM

Teiten et al., (2010) has investigated that **"CUM—The Paradigm of a Multi-Target Natural Compound with Applications in Cancer Prevention and Treatment".** In this, they worked on CUM used to target multiple intracellular components. The clinical application of the CUM was initially limited by its low solubility and bioavailability in both plasma and tissues. Its combination with the adjuvant improved the CUM's bioavailability. CUM formulation is tested for its anti-inflammatory activity and act as a potent modulator of inflammatory cell signaling. Further studies carried out its impact on tumor cell proliferation and invasion. The chemo preventive effect of CUM is mainly based on its effectiveness to inhibit tumor genesis through the decrease of cancer cell proliferation. They propose the mechanism in which cell death is induced by CUM that will hammer the tumor cell resistance to cancer treatment. CUM was used in addition with other natural or chemotherapeutic compounds that produce the synergistic effect on the drug action.

Modasiya et al., (2012) has investigated the **"Studies on solubility of CUM"** they mainly focused at increasing solubility of drug using solid dispersion technique. They prepared binary systems using different drug: polymer ratio (1:1, 1:4 and 1:8) with polyethylene glycol 4000 and 6000 by different techniques like physical mixing, melting method and solvent evaporation method. PVP K 30 was also used as a polymer. The characterization of the Formulation is carried out by scanning electron microscopy, thin layer chromatography, compatibility study, diffraction study and in vitro dissolution rate studies. The solubility of drug increased linearly with the increase in polymer concentration. They reached the net conclusion that the solid dispersion prepared by hot melt method showed higher dissolution rate in comparison to solid dispersion prepared by physical mixtures, solvent evaporation method and pure CUM.

Frawley et al., (2004) has investigated "**Nanotechnology- enhancement CUM: Symbiosis of Ancient Wisdom of East with Modern Medical Sciences review"** under which he collaborated of the possible abilities of CUM against cancer. He explored that

the CUM has the ability to work at a cellular level. CUM being a potent anti-oxidant is able to inhibit the activity of enzymes (e.g., cyclooxygenase-2 (COX-2)) responsible for cancer causing inflammation as inflammation is one of the major causes for the development of cancer as it releases powerful free radicals which both induce cell division and mutation. Secondly, CUM induces apoptosis in rapidly reproducing cancerous cell, without affecting healthy cells. Thirdly, CUM strengths the immune system and can help the body to fight off cancer. Fourthly, CUM induces angiogenesis thereby, restriction the blood supply to the cancer cells, which in turn result in death of the cells due to the starvation.

Moran et al., (2016) has investigated **"CUM and Health"** explained the anti-tumor effected on the cells explored to cancer. Cancer has important role against neurological, cancer, cardiovascular and lung diseases and also against the metabolic syndrome and liver disorders. He also explained the CUM role as nutraceutical compound.

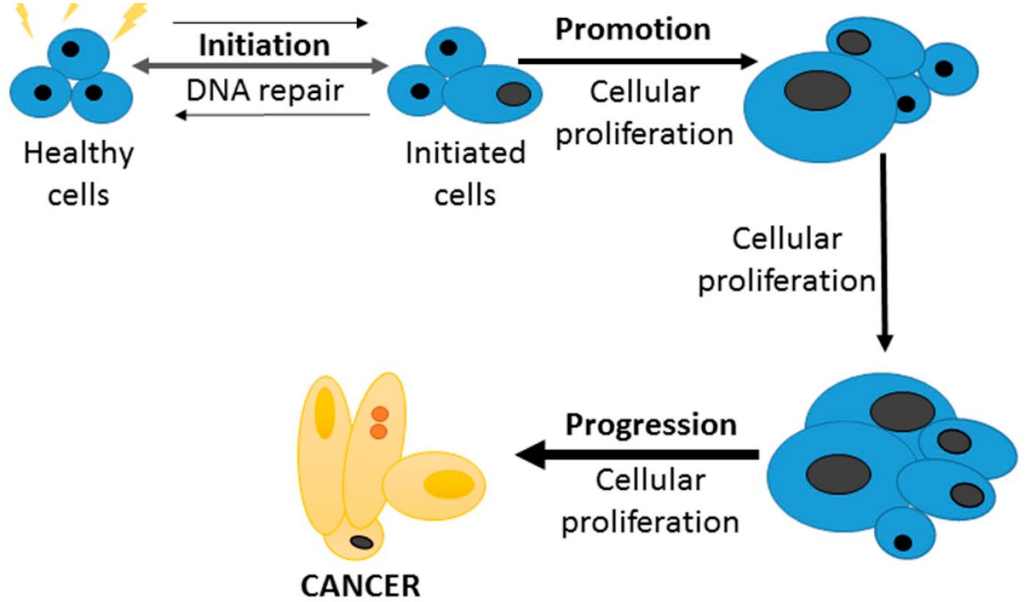


Fig. 2.2. Anti-tumor activity of CUM

(Source: Reuter et al., 2010)

Priyadarsini (2014) has investigated the **“The Chemistry of CUM: From Extraction to Therapeutic Agent”** included the methods for the extraction from turmeric, laboratory synthesis methods, chemical and photochemical degradation and the chemistry behind its metabolism. Additionally, he discussed other chemical reactions that have biological relevance like nucleophilic addition reactions, and metal chelation. He showered the lighted on to the recent advances in the preparation of new CUM nanoconjugates with metal and metal oxide nanoparticles. Directions for future investigations to be undertaken in the chemistry of CUM have also been suggested. Overall it appears the significant progress in chemistry of CUM which in turn taken as therapeutic remedy for many chronic diseases.

Sharma et al., has investigated the **“Pharmacokinetics and Pharmacodynamics of CUM”** in which they came under conclusion that CUM possesses anti-inflammatory and anti-carcinogenic properties. Many activities can be attributed to its potent anti-oxidant capacity, its diverse effect on cellular enzymes, angiogenesis and cell adhesion. In particular, they scrolled the ability of CUM to affect gene transcription and induce apoptosis in malignant cells advocates its potential in cancer chemoprevention.

3.4 Literature review on Janus emulsion

Ge et al., (2016) has investigated ***“Water–oil Janus emulsions: microfluidic synthesis and morphology design”*** in which they prepared water-oil Janus emulsion in situ by involving the use of the double-bore capillary micro fluid device. A method was elaborated in order to design the morphology of water–oil Janus emulsions. The combination of theory model and the fluids’ properties was done to symmetrically research Janus morphologies. Under the model guidance, they sensibly selected the liquids system where only the interfacial tension between the water phase and the continuous phase changed while keeping the other two interfacial tensions unchanged. They estimated that the morphology of Janus can be adjusted by changing the surfactant mass fraction in the continuous phase. In addition, with the double-bore capillary, water–oil Janus emulsions with a large flow ratio range were prepared. It involves high demand

for morphology designing of amphiphilic Janus particles by adjusting the flow ratio and the surfactant mass fraction. Amphiphilic Janus emulsions have been prepared because of their potential application as surfactants to stabilize emulsions with high controllability and reversibility. In this work, the major key point was using the numerical modeling and an experimental property that helped in designing the Janus morphologies likewise, for preparing the specific morphologies by adjusting the flow rates ratio and mass fractions of the surfactant in the outer phase in coordination with the geometry theory. This helped them to design Janus morphologies with high efficiency which in turn will help in catalysis with expensive materials.

Tu et al., (2014) has investigated the **"One-step encapsulation and triggered release based on Janus particle-stabilized multiple emulsions"** under which they carried out one-step formation of stable multiple emulsions using stimuli-responsive amiphilic Janus particle as an emulsifier. This further induced to release the encapsulants by simply increasing the pH of the continuous phase. The major area of concern was how multiple emulsions can be prepared with high stability and amphiphilic Janus particles have stimuli-responsive properties that represent a promising surfactant system to overcome this limitation. Further, using stimuli-responsive Janus particle as emulsifier has helped in destabilization of emulsions. They also showed that by changing the pH of the solution, the triggered release of the encapsulated hydrophilic species from these multiple emulsions. This study is proven as the powerful demonstration of versatility of Janus particle as solid surfactant.

Fujii et al., (2013) has investigate the **"Micrometer-Sized Gold−Silica Janus Particles as Particulate Emulsifiers"** in which they worked on to the preparations of micrometer-sized gold-silica Janus particles act as an effective stabilizer by adsorption at the oil-water interface. Hence, producing the pickering emulsion. The prepared emulsion collaborated to have both hydrophobic and hydrophilic head due to the presence of gold and silica surface. Likewise, these surfaces are exposed to the oil and water phases. In contrast, only silica particles cannot stabilize stable emulsion and complete de-emulsification occurred within 2 hrs. Also, the adsorption energy of the particles is 3 times than that of silica particles and the toxicity profile proven to be lower compared to

the conventional molecular level surfactant. They fabricated the Au-SiO2 Janus particles using vacuum evaporation. This investigation opens a new route to the preparation of the wide range of organic-inorganic hydrid microsphers.

Huanga et al., (2014) has investigated the **"Janus molecularly imprinted polymer particles"** in which they performed the control release from the Janus molecularly imprinted polymer particles. They selected [3H]-(S)-propranolol as probe in order to study the controlled drug release feature from the polymer particles. The method involved is precipitation polymerization. The grafting of the polymer layer onto MIP-core with low crosslinking has been proven to be instrumental for no effect on the template migration and the MIP-core binding ability. They involved the characterization of emulsion via FT-IR analysis, UV-visible analysis, thermogravimetric analysis, SEM analysis and radioligand binding analysis.

Indalkar et al., (2013) has investigation **"Janus Particles Recent and Novel Approach in Drug Delivery: An Overview"** in which they illustrated the Janus particles are microscopic particles composed of two different sides having a hydrophilic head group and hydrophobic tail. Janus particle are referred to as the surface-active properties and show considerably high wettability. Janus particles were synthesized by fictionalization in which particle break into fragments and then giving automatic Janus character to the fragments. They generated heterogeneous particles without control over the shape, size, or purity. Their work paid attention onto its ability to control the geometry of the Janus particles, i.e., the relative areas of their two sides and second is the ability to produce Janus particles in large quantities. They illustrated the various application of Janus particle in the field of biology sciences, nanocorals and imaging and magneto lytic theory. All such described the principles of the self-assembly process. They smartly ended up with the multivalent structure proposed in large quantities and in monodisperse form.

Chang et al., (2013) has investigated the **"Controlled Emulsion Droplet Solvent Evaporation for Particle Production"** under which they worked on the need to produce particles well-controlled size, shape and morphology for general application in catalysis, environmental remediation, nanomedicine, pharmaceutical and other fields. They

employed the emulsion droplet solvent evaporation method. In this, they carried out the synthesis of the magnetic Janus beads. And the synthesis was carried out in three different manners that include self-assembly method, masking and phase separation. The major objective was to develop and characterize high-throughput synthesis methods for particle production based on emulsion solvent evaporation techniques.

Reguera et al., (2013) has investigated the **"Advances in Janus Nanoparticles"** who focused onto the design and synthesis of Janus nanoparticles. As per the research work work undertaken shows Janus nanoparticles are highly versatile nanomaterial with great potential in Scientific and technological fields. He involved various techniques involved in identification of Janus nanoparticles such as electron microscopy and scanning probe microscopy, and macroscopic methods such as 2D NOESY NMR, contact angle, *etc*. He incorporated the various methods involved in synthesis of the Janus particles:

- Methods that uses masks to modify one part of the NPs.
- Methods based on controlled nucleation at NP surfaces.
- Methods based on the phase separation of multicomponent symmetric NPs.
- Methods based on the self-assembled process

He also worked on making familiar with the properties and applications of Janus nanoparticles. Property such as amphiphilicity makes them excellent candidates for interface-related applications such as the stabilization of emulsions through porous membranes.

3.5 Literature review on Skin carcinoma

Veronique et al., (2008) has investigated the "**Melanoma-Part 1: epidemiology, risk factors and prevention**" and came out with the brief description on how common the melanoma is and one must be concern about its rising incidence. He characterized melanoma according to the thickness and histological subtypes. The resulted scenario was that the thinner tumors were seen in younger individuals whereas nodular melanoma

and lengtigo maligna melanoma tend to affect older individuals. He also determined the area usually effected in melanoma and according to the sex of the individual, the most common site for women is the legs and for men the truck. The various risk factors involved in melanoma are being underlined and various preventive measures taken in order to decrease the risk of the melanoma.

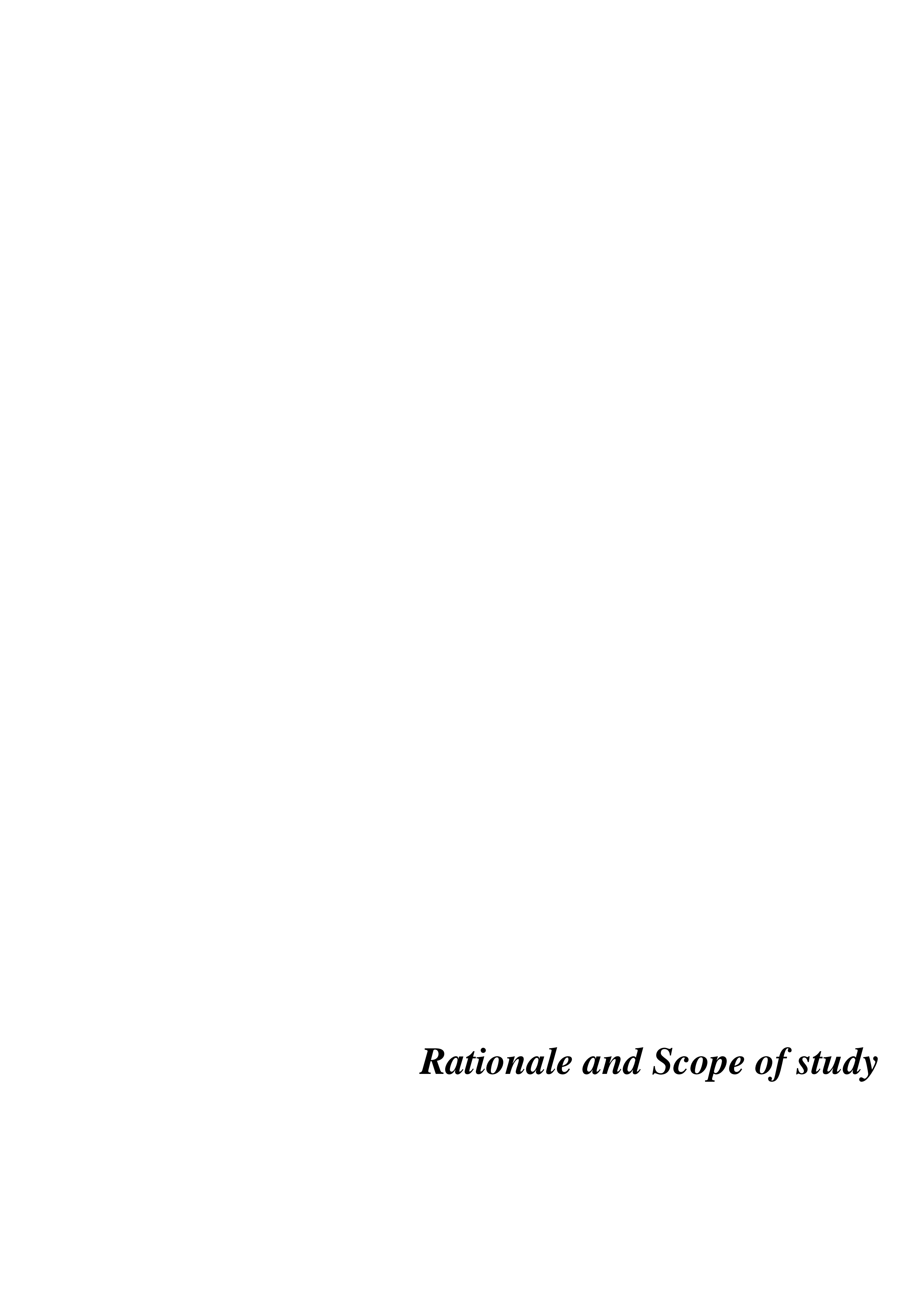
Garbe et al., (2016) has investigated the "**Diagnosis and treatment of Melanoma. European consensus-based interdisplinary guideline**" came out on brief introduction to melanoma namely, Cutaneous melanoma and determined it to be the most dangerous form of skin tumor and causes 90 % of skin cancer mortality. The upshot of safety margin of melanoma is exercised with 1-2 cm. he came out with the investigation that the patient suffering with stage II and III melanoma can be treated with the Interferon- alpha as an adjuvant therapy. He resolved for the systemic treatment of the melanoma chemotherapy is the only way and may be considered in the second and third line patients with resistance to immunotherapy and targeted therapy. In immunotherapy, cytokines are examined several times in non-controlled clinical trials and improvement of survival was randomized. He established the fact that surgical excision is the only primary treatment of melanoma.

Giacomamtonio et al., (2013) has investigated "**Guidelines for the management of Malignant Melanoma**" and has his major focus on the Nova Scotia approach to melanoma. His major goals were to establish a diagnosis and to comment on the relevant prognostic factors. He was mainly concerned with the thickness of the lesion, the presence or absence of ulceration, the mitotic rate in thin melanoma, the presence or absence of microsatellites and the status of surgical margins. For him the challenging work was the microscopic interpretation of pigmented lesions. For such microscopic evaluation of pigmented skin lesions require a consistent and systematic procedural approach. Also, the vertical thickness of the primary tumor is the most important factor in determining the nonmetastatic melanoma. For the early detection of the melanoma, seven point checklists have been validated as a screening tool which includes the major factors and the minor features. He shortlisted the major features as change in size, color, shape

whereas, the minor features which may increase the suspicion of melanoma include its diameter, sensory change, inflammation, oozing, crusting and bleeding.

Silpa et al., (2013) has investigated the "**A review on skin cancer**" where he resolute the types of the skin cancer and the major cause of occurrence of skin melanoma. Firstly, he illustrated that the major cause of the occurrence of the melanoma is the sun exposure and other factors include the ozon depletion and chemical exposures of the skin that are involved in the precipitation of the skin cancer. Basically, the mutation of the P53 gene is involved in UV-induced carcinogenesis and P53 gene acts vital in development of Squamous cell carcinoma. So, he deduced that the less exposure to the UV radiations will dethrone the occurrence of the skin carcinoma. The application of the sunscreens is the primary prevention of the ongoing increase rate of the melanoma. He mainly proposed the review on the outline of various types of melanoma, its pathogenesis, diagnosis, prevention and treatment of the skin cancer.

Bandarchi et al., (2010) has investigated "**A review article on from melanocytes to metastatic malignant melanoma**" concluded that Malignant melanoma is the most aggressive malignancies in human and is responsible for almost 60 % of the lethal skin tumors. From the past two decades, its incidence has been increasing in white population. He also deduced that there is a complex interaction of environmental (exogenous) and endogenous, including genetic, risk factors, clinical presentations, historical classification and histopathology. He carried out recent discoveries in biology and genetics of malignant melanoma, including its classification.



Rationale and Scope of study

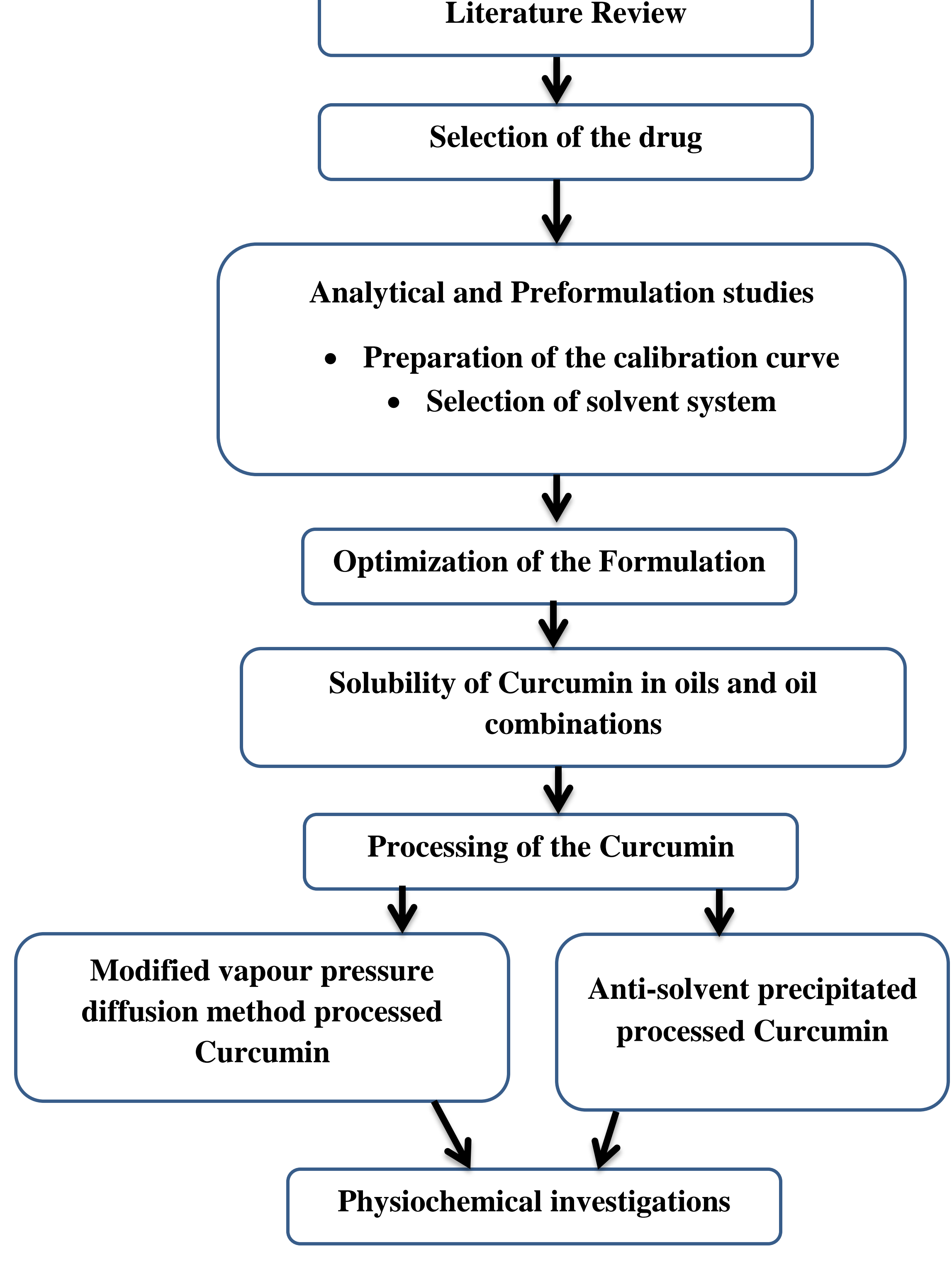
CHAPTER 4

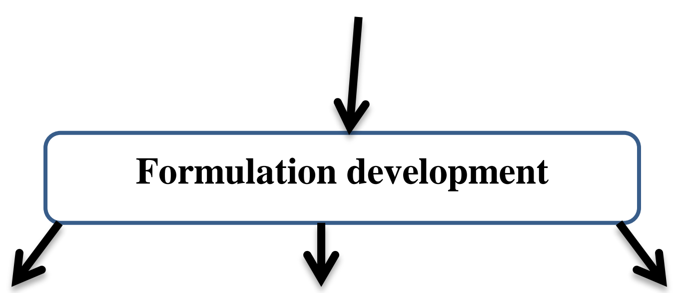
RATIONALE AND PLAN OF WORK

4.1 Rationale of Work

In pharmaceutical technology, incorporation of both hydrophilic and hydrophobic drug molecules together with in a single drug delivery system for the management of a particular/specific drug condition is always a challenging as far as formulation scientist point of view. An unexpected bi-compartmental structure as observed with the currently developed Janus emulsion systems try to provide a plausible solution for the above set problem. It is particularly true for the disease like melanoma, which requires combined drug delivery for its effective management. Asafoetida and CUM possess multi-functional therapeutic activities including anti-cancer effect. Majority of the experimental works shown in the current dissertation work focused only on processed and unprocessed CUM powders and Janus emulsion containing processed and unprocessed CUM. Following three different forms of CUM to keep inside the Janus emulsion systems, the *in vitro* drug release, *in vitro* antiinflammatory effect, *in vitro* antioxidant effect and *in vitro* haemolysis test were performed in comparison with pure CUM powder or reference drug product. At the rudimentary stage of the work, the asafoetida powder was also incorporated into the CUM loaded Janus emulsions to see initially via compound microscopic technique, the possibility of entrapping these two drugs together in emulsion systems. The presence of unique bi-compartmental structure of the dispersed oil droplets of the emulsion actually allowed the entrapment of other drug moiety with different drug solubility characteristics.

4.2 Plan of wok





Formulation development

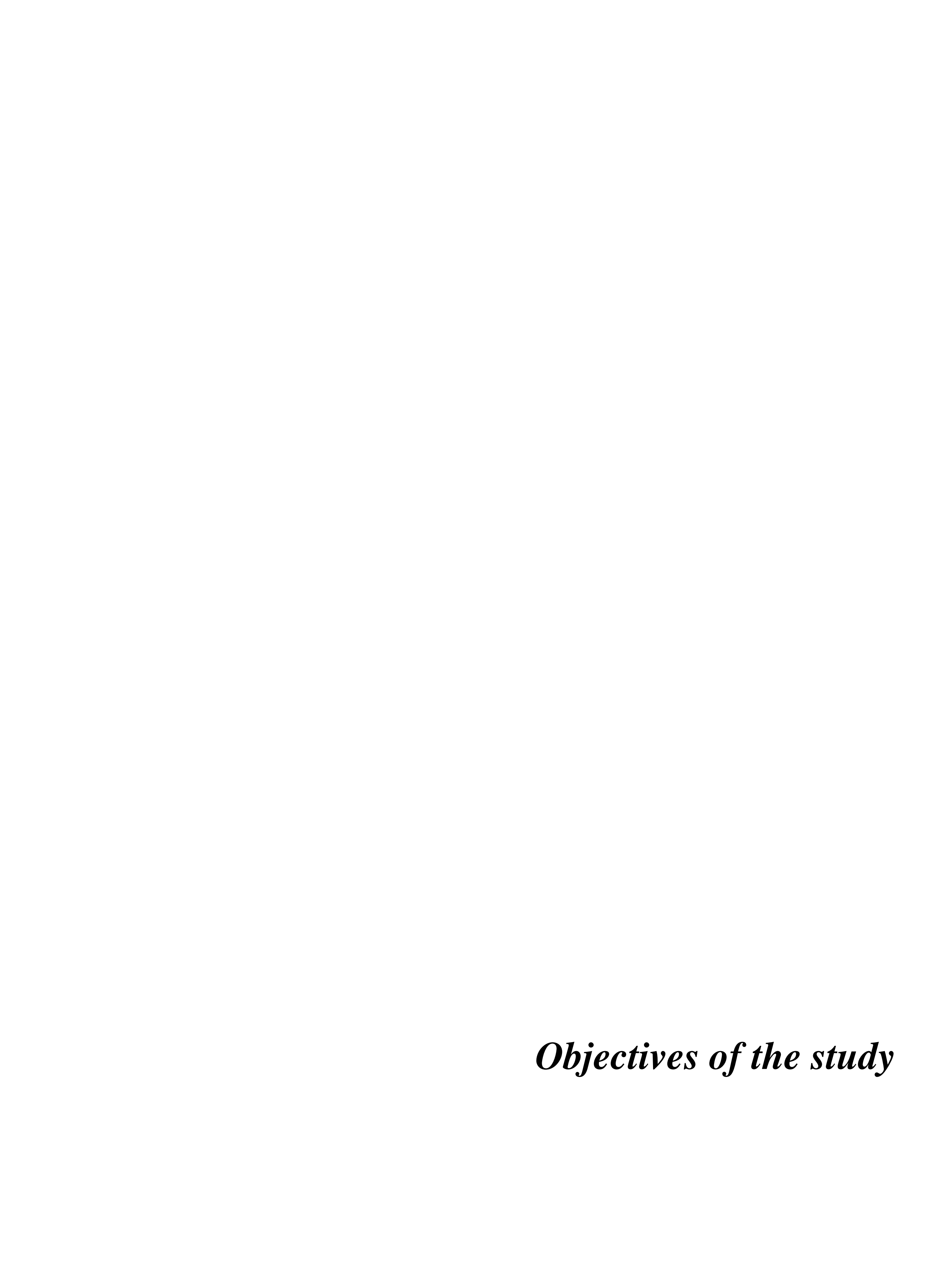
Janus emulsion containing unprocessed Curcumin

Janus emulsion containing modified vapour pressure diffusion method processed Curcumin

Janus emulsion containing anti-solvent precipitated processed Curcumin

- **Particle size analysis by microscopy**
- **% drug entrapment efficiency and drug amount at o/w interface**
- **TEM analysis**
- **TLC determination**
- ***In vitro* drug release**
- ***In vitro* antiinflammatory activity**
- ***In vitro* antioxidant activity**
- **% Haemolysis test**

Submission of report



Objectives of the study

CHAPTER- 5

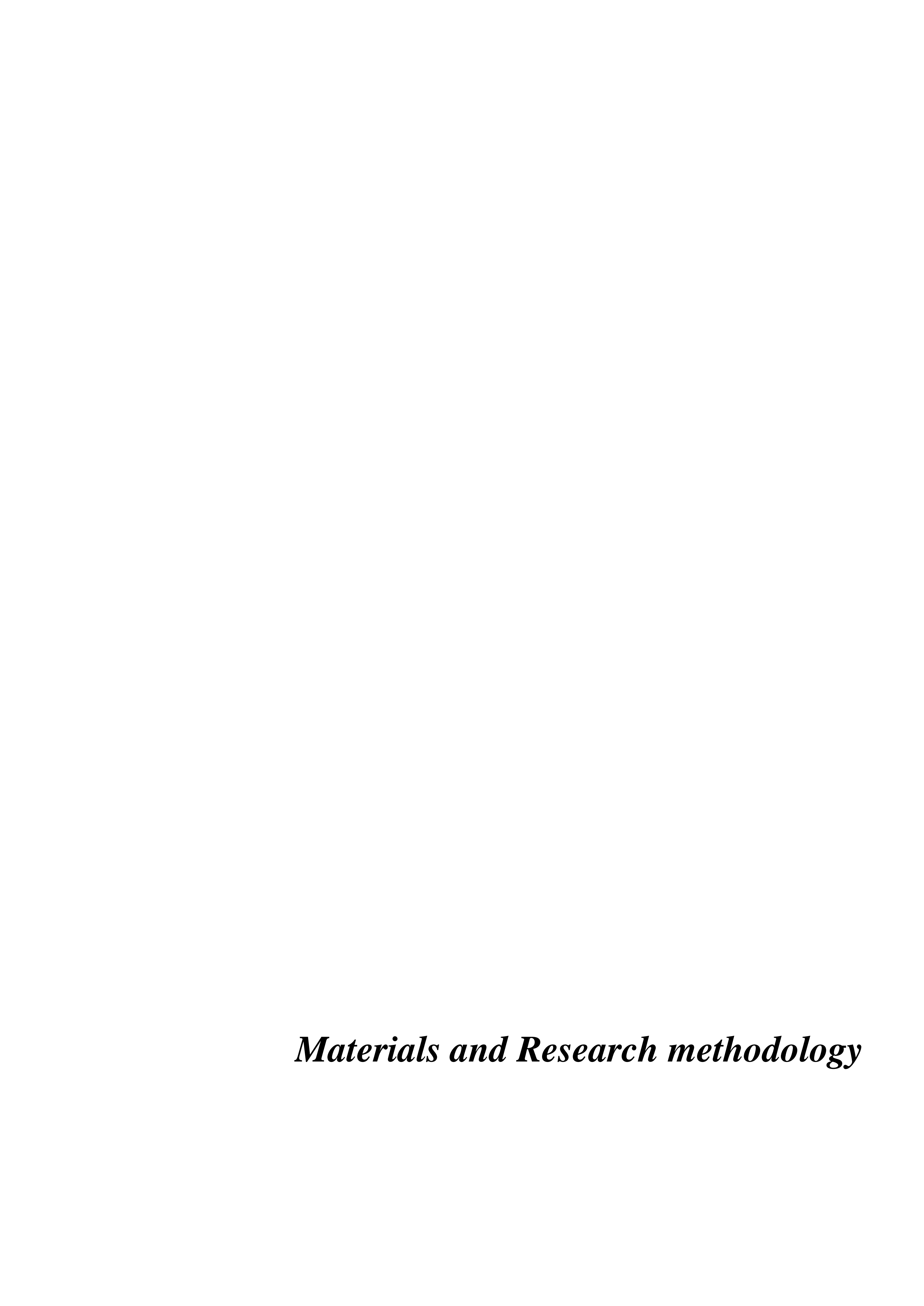
OBJECTIVES OF THE STUDY

5.1 Aim

The aim of current investigation is to design, develop and perform few *in vitro* evaluation tests of processed and unprocessed CUM-loaded oil-in-water topical Janus emulsions.

5.2 Objectives of work

- To see the solubility of CUM in various organic solvents, oils and oil combinations
- To prepare topical Janus emulsion formulation containing dispersed oil droplets in micron sized particles
- To see the possibility of producing dispersed oil droplets with double-head faced structure (Janus particle)
- To determine the drug entrapment efficiency as well as the drug positioning at oil-in-water interface
- To analyze the internal structure of the dispersed oil droplets of the Janus emulsion via TEM analysis
- To calculate the *in vitro* drug release in DMSO and pH 6.8 phosphate buffer (7:3) from Janus emulsion over the time period of 2 hours 30 minutes
- To find out the potential of free radical scavenging activity of CUM (processed and unprocessed powder) and Janus emulsion containing processed and unprocessed CUM via DPPH study
- To calculate the *in vitro* antiinflammatory activity of Janus emulsion containing processed and unprocessed CUM via protein denaturation assay
- To estimate the toxicity of CUM powder (unprocessed and processed) and Janus emulsion containing processed and unprocessed CUM via RBC breakdown assay
- To see the possibility of entrapping hydrophobic CUM and hydrophilic asafoetida together in the Janus emulsion developed



Materials and Research methodology

CHAPTER 6

MATERIALS AND RESEARCH METHODOLOGY

6.1 Materials

6.1.1 Chemicals used

Table 6.1 List of various materials used in current project

Chemical Name	Supplier
Ascorbic acid	*Akhil Heathcare Pvt. Ltd., Mumbia, India*
Castor Oil	*Khurana Oils, Ludhiana, India*
Coconut Oil	*Khurana Oils, Ludhiana, India*
DEE	*Vee Dee Enterprises, Mumbai, India*
Diclofenac Sodium	*Kwality Pharma, Amritsar, India*
DMSO	*Loba Chemie, Mumbai , India*
DPPH	*Sisco Research laboratories, Mumbai, India*
EDTA	*Teilwara, Delhi, India*
Egg Albumin	*Loba Chemie, Mumbai, India*
Ethanol	*Solvchem, Mumbai, India*
Methanol	*Loba Chemie, Mumbai, India*
Olive Oil	*Khurana Oils, Ludhiana, India*
Potassium di hydrogen orthophosphate	*Loba Chemie, Mumbai, India*
Silicone Oil	*Loba Chemie, Mumbai, India*
Sodium Hydroxide	*Loba Chemie, Mumbai, India*
Tween 20	*Niram Chemicals, Mumbai, India*
Tween 80	*Loba Chemie, Mumbai, India*

6.2 Equipments Used

Table 6.2 List of various equipments used in current project

Equipments	Suppliers
Centrifuge	Remi Electrotechnik Ltd. Vasai, India
Double Beam UV- Visible Spectrophotometer	Shimadzu 1800 Japan
Fourier transform Infrared Spectrophotometer	Shimadzu FTIR-8400 S Japan
Hot Air Oven	Navyug, Punjab, India
Incubator	Remi
Magnetic Stirrer	Remi Motors Pvt. Ltd. Vasai, India
Mechanical water bath shaker	Remi Motors Pvt. Ltd. Vasai, India
Melting Point Apparatus	Popular Traders, Ambala Cantt. , Ind
Microscope	Kyowa Getner, Japan
pH Meter	Labtronics, Punjab , India
Refrigerator	Kelvinator ,USA
Rota evaporator	IKA HB 10
Transmission Electron Microscope	FEI Tecnai, Netherlands
Ultrasonicator	Athena Technology, India
UV visualizer	Perfit India
Weighing Balance	Shimadzu, Japan

6.2 Research methodology

6.2.1 Physical description

The sample of CUM was identified and characterized as per requirements of COA (certificate of analysis) issued by the manufacturer and (USP 30 NF 25, 2007).

6.2.2 Melting point analysis

The melting point of CUM (unprocessed and processed as mentioned in 6.4.1 and 6.4.2 methods modified vapour pressure diffusion method and anti-solvent precipitated method) was determined using capillary tube method. In this method, the double-end opened capillary tube was taken and the one end was sealed using direct flame. Then, the drug was filled in the capillary tube before inserting it into the melting point apparatus. The transformation of solid state drug particles into liquid state was noted and corresponding temperature at which this solid to liquid transformation occurred was also noted down.

6.2.3 Fourier transform infra-red (FT-IR) spectroscopy

The most well-known method of preparing the pellet my mixing the drug particle and KBr in the ratio of 1:40 was used to find the FT-IR spectra of CUM. The functional groups present in the drug molecule structure were scanned between the wave number values of 600-6000 cm^{-1} using a FT-IR spectrophotometer.

6.2.4Analytical method development

6.2.4.1 Selection and Optimization of Solvent

The selection of solvent has a profound influence on the quality and shape of the peak. For UV method development, the major solvents used are: Chloroform, Acetone, Methanol, DMSO, Phosphate buffer, ethanol, water etc. Under this step, different solvents were optimized. Out of which Methanol satisfied all the conditions relative to peak quality and non-interference at specified wavelength.

6.2.4.2 Determination of wavelength maxima

6.2.4.2.1 Determination of wavelength maxima using methanol as solvent

CUM 5 µg/mL solution was scanned in UV spectrophotometer in range of 200-800 nm. Methanol is used as blank. Wavelength corresponding to maximum absorbance of CUM in methanol was observed at **419 nm**.

6.2.4.2.2 Determination of wavelength maxima using DMSO as solvent

CUM 5 µg/mL solution was scanned in UV spectrophotometer in range of 200-800 nm. Methanol is used as blank. Wavelength corresponding to maximum absorbance of CUM in methanol was observed at **422 nm**.

6.2.4.2.3 Determination of wavelength maxima using DMSO and pH 6.8 phosphate buffer (7:3) as solvent

CUM 5 µg/mL solution was scanned in UV spectrophotometer in range of 200-800 nm. Methanol is used as blank. Wavelength corresponding to maximum absorbance of CUM in methanol was observed at **558 nm**.

6.2.5 Preparation of standard calibration curve

6.2.5.1 Calibration curve in methanol (λmax = 419 nm)

The standard calibration curve of CUM was obtained by measuring the absorbance of CUM solution in concentration range (1-5 µg/mL) prepared from stock solutions in methanol at 419 nm in triplicate. The calibration curve of CUM was then plotted with absorbance on y-axis and CUM concentration (µg/mL) on x-axis.

6.2.5.2 Calibration curve in DMSO (λmax = 424 nm)

The standard calibration curve of CUM was obtained by measuring the absorbance of CUM solution in concentration range (1-5 µg/mL) prepared from stock solutions in DMSO at 424 nm in triplicate. The calibration curve of CUM was then plotted with absorbance on y-axis and CUM concentration on x-axis.

6.2.5.3 Calibration curve in DMSO and pH 6.8 phosphate buffer (λmax = 558 nm)

The standard calibration curve of CUM was obtained by measuring the absorbance of CUM solution in concentration range (1-5 µg/mL) prepared from stock solutions in methanol at 558 nm in triplicate. The calibration curve of CUM was then plotted with absorbance on y-axis and CUM concentration on x-axis.

6.2.6 Validation of calibration curve

Validation is defined as establish documented evidence which provides a high degree of assurance that a specific activity will consistently produce a desired result meeting its predetermined specifications and quality characteristics. Validation includes several parameters like linearity, accuracy, precision, ruggedness and robustness, limit of detection (LOD) and limit of quantification (LOQ) as per ICH guidelines.

6.2.6.1 Linearity and Range

Linearity can be determined by taking the drug concentration in between 1-5 µg/ml. it is determined by taking aliquots from stock solution of CUM of 1 mg/ml. all the diluted solution were scanned 200-800nm using methanol as blank. The calibration curve was plotted on graph of Concentration vs. absorbance to check the linearity [ICH Q2 (R1)].

Acceptance criteria

Correlation Coefficient ≥ 0.9991

6.2.6.2 Precision

The precision is determined by assay of five determinations at 100% test concentration (20ppm) and relative standard deviation (% RSD) was calculated. The results were reported in terms of % relative standard deviation [ICH Q2 (R1)].

Acceptance Criteria

The Relative Standard Deviation should not be more than 2%.

6.2.6.3 Accuracy

The accuracy of an analytical method was determined using the samples in which known amount of analyte is added. The accuracy is calculated from the results by calculating the percentage of analyte recovered using the assay at three different levels i.e. 50 %, 100 % and 150% of the method concentration (200 µg/mL) [ICHQ 2 (R1)]. A standard stock solution 1 mg/ml of CUM was prepared. In volumetric flasks of 10 mL, aliquots of 0.15, 0.3 and 0.45 mL of this solution (which would yield concentrations of 15, 30 and 45 µg/mL respectively) were combined with 1.5 ml of the 200 µg/mL sample solution in 10 mL volumetric flask and volume make up done using methanol. Thus the final concentrations were 45.0, 60.0 and 75 µg/mL, which correspond to 50, 100 and 150% of the target concentration, respectively.

The sample was prepared in triplicate and analysed by using UV-Vis spectrophotometer at 419 nm.

Acceptance Criteria

Mean recovery = 98 to 102%

6.2.6.4 Robustness

CUM (1-5 µg/mL) solution was analysed six times at two different temperatures (18 ^{o}C and 24 ^{o}C) to determine robustness of the method. The result is indicated as % RDS.

6.2.6.5 Ruggedness

Ruggedness was determined by analysing 1-5 µg/ml concentration solution in methanol by two analysts at 419n nm. The result is indicated as % RSD.

6.2.6.6 Limit of detection

"It is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions". Limit of detection is calculated using following equation.

$$\mathbf{LOD = 3.3\ X\ \frac{\sigma}{S}} \qquad \textbf{eq. (1)}$$

Where,

- σ is standard deviation of the response
- S is slope of the calibration curve

6.2.4.6 Limit of quantification

"It is the lowest concentration of analyte in a sample that can be determined with the acceptable precision and accuracy under stated experimental conditions." Limit of quantification is calculated using following equation.

$$\mathbf{LOQ = 10\ X\ \frac{\sigma}{S}} \qquad \textbf{eq. (2)}$$

Where,

- σ is Standard deviation of the response
- S is Slope of the calibration curve (Mastiholimath et.al, 2012)

6.2.7 Solubility of CUM in various solvents, oils and oil combinations

The solubility of CUM was determined in presence of various oils, organic solvents and oil combinations such as castor oil, coconut oil, DMSO, ethanol, olive oil, methanol, phosphate buffer solution of pH 7.4, silicone oil, sodium hydroxide and water. Excess amount of CUM was added in the selected oils, solvents or oil combinations and all the samples were shaken for four hours using a mechanical water bath shaker. All the samples were filtered and the filtrates after suitable dilution with methanol were measured at 419 nm spectrophotometrically.

6.2.8 Formulation Development

6.2.8.1 Ingredients used in the preparation of Janus emulsion

The various ingredients used in the preparation of Janus emulsion were described briefly below.

6.2.8.1.1 Silicone oil

The term silicone is derived from the mnemonic acronym such as silicone, oxygen and methane. The British scientist during the yearly 1900 coined the term silicone to describe a large family of synthetic polymers containing elemental silicone. The physical nature of silicone is depending on the function of polymerization and cross-linkage of polymers (silicone, oxygen and ethane). The silicone polymers can exist as solids (elastomers), gels and free flowing liquids. The viscosity of silicone fluid is measured in centistokes (cS), with 100 cS being the viscosity of water. Currently, two silicone oils (AdacoSil 5000 and SiliKon 1000) are approved by the US-FDA for use in the eye for severe retinal detachment and therefore, these two silicone oils are used in oil during eye surgery to prevent Rorfet detached retina. All other uses of the silicone oil maintained in the literature are simply "off-labelled". The meaning of off-label is the medicinal use other than approved or other then indicated.

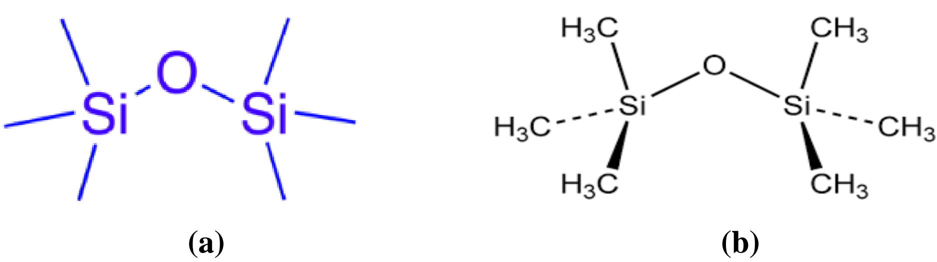
For over 5 decades, liquid injectable silicone has been used for soft-tissue augmentation. In many ways, liquid silicone appears to fulfil most of the criteria to be used as an ideal implantable substance. For example, following implantation of approximately 0.01 mL, the silicone oil is permanent, non-carcinogenic, and minimally antigenic and does not support bacterial growth. Technically, Silicone oil is unaffected by exposure to sunlight and most chemicals. Furthermore, silicone oil can easily be heat sterilized. Moreover, its viscosity remains constant throughout the range of temperatures experienced by patients.

The scientific name for liquid silicone is dimethylpolysiloxane fluid. Unfortunately, intravenous injections of silicone-based products must be scrupulously avoided.

The silicone was intentionally adulterated with various formulas including Sakarai formula, to increase inflammation and the fibroplasia that resulted following injection to silicone products into human body. The Sakarai formula is the mixture of silicone and olive oil. This intentional adulteration followed publication in various

journals that suggested that silicone was non-toxic even when adulterated (Kagan et al., 1963; Selmanowitz et al., 1997 and Rees et al., 1965).

Siloxane (Si-O-Si) is functional group in organosilicon and silicone oil is polymerised siloxane with organic side chains. The production of amino-modified silicone oil was carried out with dimethyl cyclosiloxane (DMC) and N-(2-aminoethyl)-3-aminopropyl methyldimethoxysilane (KH-602) as raw materials and potassium hydroxide (KOH) as a catalyst and 1,3-Diethenyl-1,1,3,3-tetramethyl disiloxane as a cured agent. The most important member of silicone oil is polydimethylsiloxane. These are of high thermal stability and lubricating properties.



(a) **(b)**

Fig 6.1: Structure of (a) Siloxane (b) Silicone oil

There are numerous types of silicone oil. Based upon the cyclic structure, these are classified as cyclosiloxanes and non-cyclic siloxane liquids, which show relatively high volatility and used as antiperspirant in cosmetic products. Others include simethicone that are potent anti-foaming agent due to its low surface tension (Moretto et al., 2005).

Table 6.3 Properties of silicone oil

Physical and chemical properties of silicone oil	
Physical state	Liquid
Colour	Clear, colourless
Odour	Weak odour
Viscosity	500 cP at 25 ˚ C
Vapour pressure	<1 mm Hg at 20 ˚ C

Boiling point	>140 ˚C at 760 mm Hg
Solubility in water	Insoluble
Specific density	1.09 g/mL at 20 ˚ C
Chemical stability	Stable under normal temperature and pressure
Molecular formula	$C_6H_{18}OSi_2$
Molecular weight	162.380 g/mol
Incompatibility	Strong oxidising agent

Silicone oil is regarded as power chemical under ISO9001 ISO14001 certified (www.SiSiB.com)

Suppliers: Company: Santa Cruz Biotechnology, Inc. 2145 Delaware Ave Santa Cruz, CA 95060 Telephone: 800.457.3801 or 831.457.3800.

6.2.8.1.2 Olive oil

Olive oil is the liquid fat obtained from the olives (the fruit of *Olea europaea*; family *Oleaceae*).it is basically used as in cosmetics, pharmaceuticals and soaps and fuel for oil lamps. It consists of oleic acid (83%), small amount of other fatty acids including linoleic acid (21%) and palmitic acid (20%).

Structure of olive oil

Fig. 6.2: Chemical structure of olive oil

Uses of olive oil:

- Used as cooking oil in countries surrounded the Mediterranean
- Used as a salad dressing
- Natural and safe lubricant to be used in health care products

- Used as both solvent and ligand in synthesis of cadmium selenide quantum dots

Table 6.2 Properties of olive oil

Physical and chemical properties of olive oil	
Physical state	Liquid, floats on water
Colour	Clear, pale yellow colour
Odour	mild odour
Viscosity	84 cP at 25 ˚ C
Boiling point	>700 ˚C at 760 mm Hg
Refractive index	1.467-1.470
Specific density	0.911 g/mL at 20 ˚ C
Chemical stability	Stable under normal temperature and pressure
Molecular formula	$C_{18}H_{34}O_2$
Molecular weight	282.468 g/mol
Saponification value	184-196

(National Center for Biotechnology Information, U.S. National Library of Medicine, 8600 Rockville Pike, Bethesda, MD20894, USA)

6.2.8.1.3 Coconut oil

It is edible oil obtained from the coconut palm (*Cocos nucifera*, family: *Arecaceae*). It has various applications in food, medicine and industry. It is insoluble in water. It has many beneficial effects in heart diseases as it contains 50% lauric acid, used in various heart problems like high cholesterol levels and high blood pressure. Coconut oil is also beneficial in weight loss.

Chemical structure of coconut oil

Fig 6.3: Chemical structure of coconut oil

Table 6.5 Properties of coconut oil

Physical and chemical properties of coconut oil	
Physical state	Liquid, floats on water
Colour	Colourless at 30 ˚ C
Odour	Bleached and deodorized
Viscosity	84 cP at 25 ˚C
Specific heat	1.910 J/g
Melting point	25 ˚C
Refractive index	1.465-1.467
Specific density	0.914 g/mL at 20 ˚C
Chemical stability	Stable under normal temperature and pressure
Molecular formula	$C_4H_8NNaO_2$
Molecular weight	125.103 g/mol
Thermal conductivity	0.179-0.188
Smoke point temperature	232 ° C

(National Centre for Biotechnology Information, U.S. National Library of Medicine, 8600 Rockville Pike, Bethesda, MD20894, USA)

Uses of Coconut oil

- Used in cooking as it has high smoke point temperatur
- Used in making margarine, soap and cosmetics
- Used as skin moisturiser and help in healing Keratosis pilaris
- Used for cooling and soothing effect on skin

6.3.1.4 Tween 80

Tween 80 is a non-ionic surfactant and emulsifier used in food and cosmetics.

Structure of Tween 80

Fig. 6.4: Chemical structure of Tween 80

Table 6.6 Properties of Tween 80

Physical and chemical properties of Tween 80	
Physical appearance	Viscous, water soluble yellow liquids
HLB value	15.0
Chemical formula	$C_{32}H_{60}O_{10}$
Molecular weight	604.822 g/mol
Molar mass	1310 g/mol
Density	1.06-1.09 g/mL
Boiling point	>100 ˚C
Solubility in water	Very soluble
Solubility in other solvents	Soluble in ethanol, cottonseed, corn oil, toluene
Viscosity	300-500 centistokes
Critical micelle concentration (CMC)	0.012mM
Main hazard	Irritant

6.2.9 Preparation of processed CUM

6.2.9.1 Processing by Modified vapour pressure diffusion method

The previously reported modified vapour pressure diffusion method (Mittal et al., 2016) was simply followed to make the processed CUM particles.

In this method, 500 mg of CUM was accurately weighed and dissolved in 5ml of methanol in a 50-ml capacity beaker to prepare a supersaturated solution. This beaker was then placed in a 250-ml capacity beaker which was already filled with 30 ml of diethyl ether (DEE), a non-solvent. Extreme care should be taken such that the two beakers don't touch each other. The whole set up was tightly closed from the top using aluminium foil, and heated at the temperature of 35˚C. After the complete evaporation of DEE from 250 ml of beaker, the solution that remained in the 50-ml beaker was then placed in refrigerator (3 - 4 ˚ C) for 12 h to obtain the CUM crystals. The product was filtered and dried in hot air oven at 40 ˚ C for 10 mins.

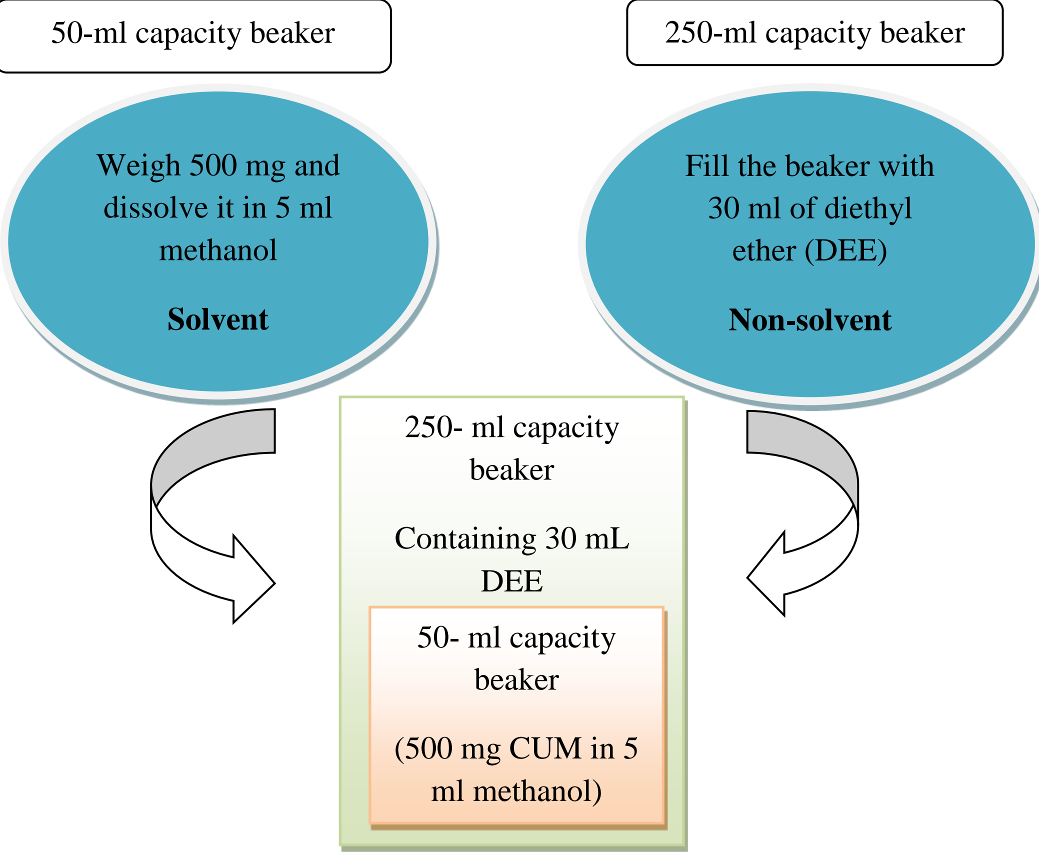


Fig 6.5: Set up of modified vapour pressure diffusion methanol

6.2.9.2 Processing by anti-solvent precipitation technique

The technique adopted by Aditya et al., (2017) was followed to prepare the processed CUM particles. The process involved the usage of food products that are CUM, methanol, double distilled water.

Here two solvents were used; the first solvent is methanol in which CUM is readily soluble (5.862 mg/ml). This was labelled as the solvent phase. The second solvent was taken as water in which CUM is sparingly soluble (0.34 mg/ml) and this was labelled as antisolvent. Also, a drop of Tween 80 was added as stabilizer. Else, pure water was used as antisolvent.

Initially, CUM was dissolved in the ethanol (solvent) 500 mg in 10 ml ethanol. The CUM solubilized ethanolic solution was then mixed with water (anti-solvent). The both solvents were mixed in 1:5. The solvent was evaporated from the mixture of solvent and antisolvent using rotary evaporator under vacuum condition (-90kPa) by rotating the round bottom flask at 150 rpm for 10 min. the temperature of the water bath is maintained at 45˚C. Solvent evaporation resulted in supersaturation of CUM in antisolvent, which lead to its precipitation (nucleation).

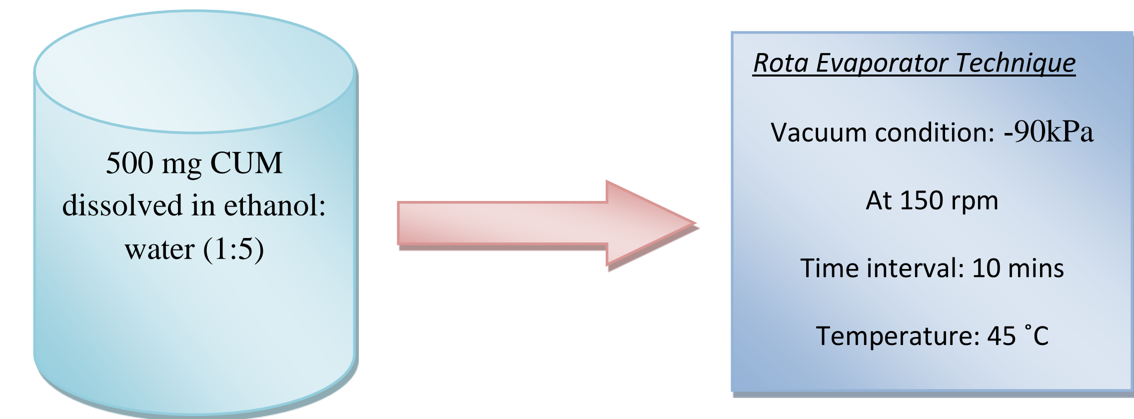


Fig. 6.6: Set-up of Anti-solvent precipitation technique

6.2.10 Formulas used in the preparation of Janus emulsion

The ingredients used to prepare both blank-and CUM loaded-microparticles are shown in Tables 6.4 with formulation notations F_1, F_2, F_3,………… F_{13}.

Table 6.7 Composition of blank Janus emulsions for initial formulation development work

Formulation	Water phase		Oil phase	
	Water (g)	Tween 80 (g)	Olive oil (g)	Silicon oil (g)
F_1	9.782	0.408	1	2
F_2	19.564	0.408	1	4
F_3	19.564	1	1	2
F_4	39.128	2	1.67	3.33
F_5	39.128	2	1.67	3.33
F_6	39.128	3	1.67	3.33
F_7	39.128	4	1.67	3.33
F_8	39.128	5	1.67	3.33
F_9	39.128	5	1	4
F_{10}	39.128	5	1.25	3.75
F_{11}	39.128	5	0.56	4.44
F_{12}	9.782	5	1	3
F_{13}	19.564	5	1	3

Table 6.8 Composition of formulation considered for further research work (F_{14})

CUM (mg)	Water phase		Oil phase	
	Water (g)	Tween 80 (g)	Olive oil (g)	Silicon oil (g)
40	19.56	0.408	1	4

6.2.11 Preparation of Janus emulsion

- Take a 100 ml beaker; put required quantity of water and Tween 80
- The above prepared water solution needs to be solubilize gently to avoid bubble formation and maintain the temperature to 70°C
- Then, take another 50 ml beaker; add required quantity of Silicon oil and olive oil
- Stir the above prepared oil solution on magnetic stirrer
- Gently, mix oil solution to water solution
- Keep it on magnetic stirrer for 15 min. Then, further on for homogenous mixing on the mechanical stirrer for half an hour

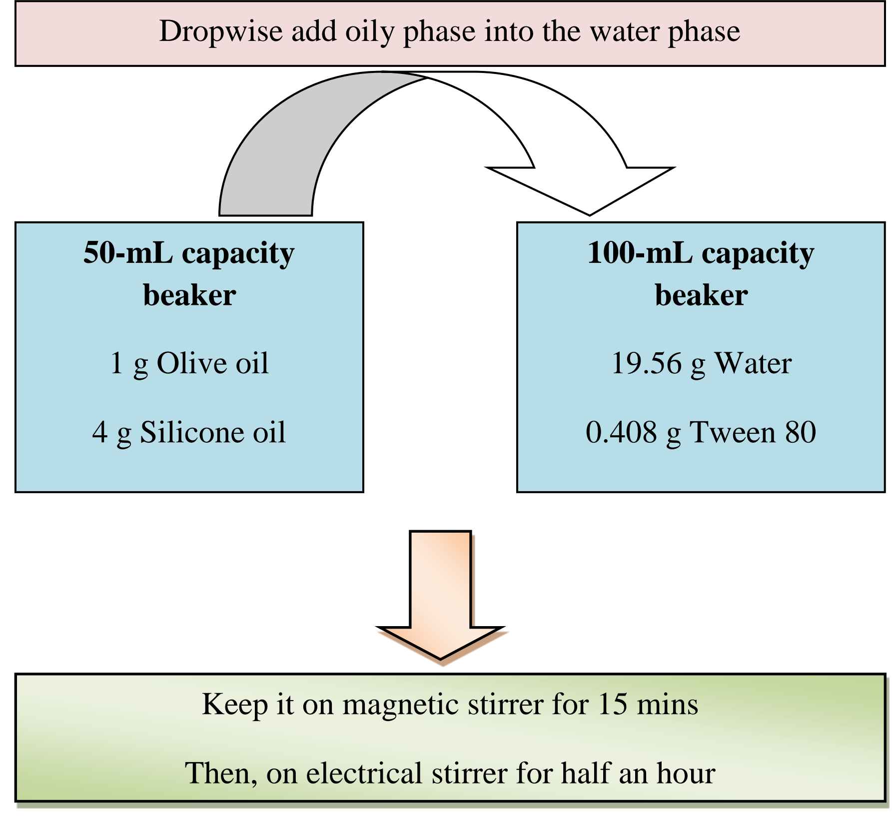
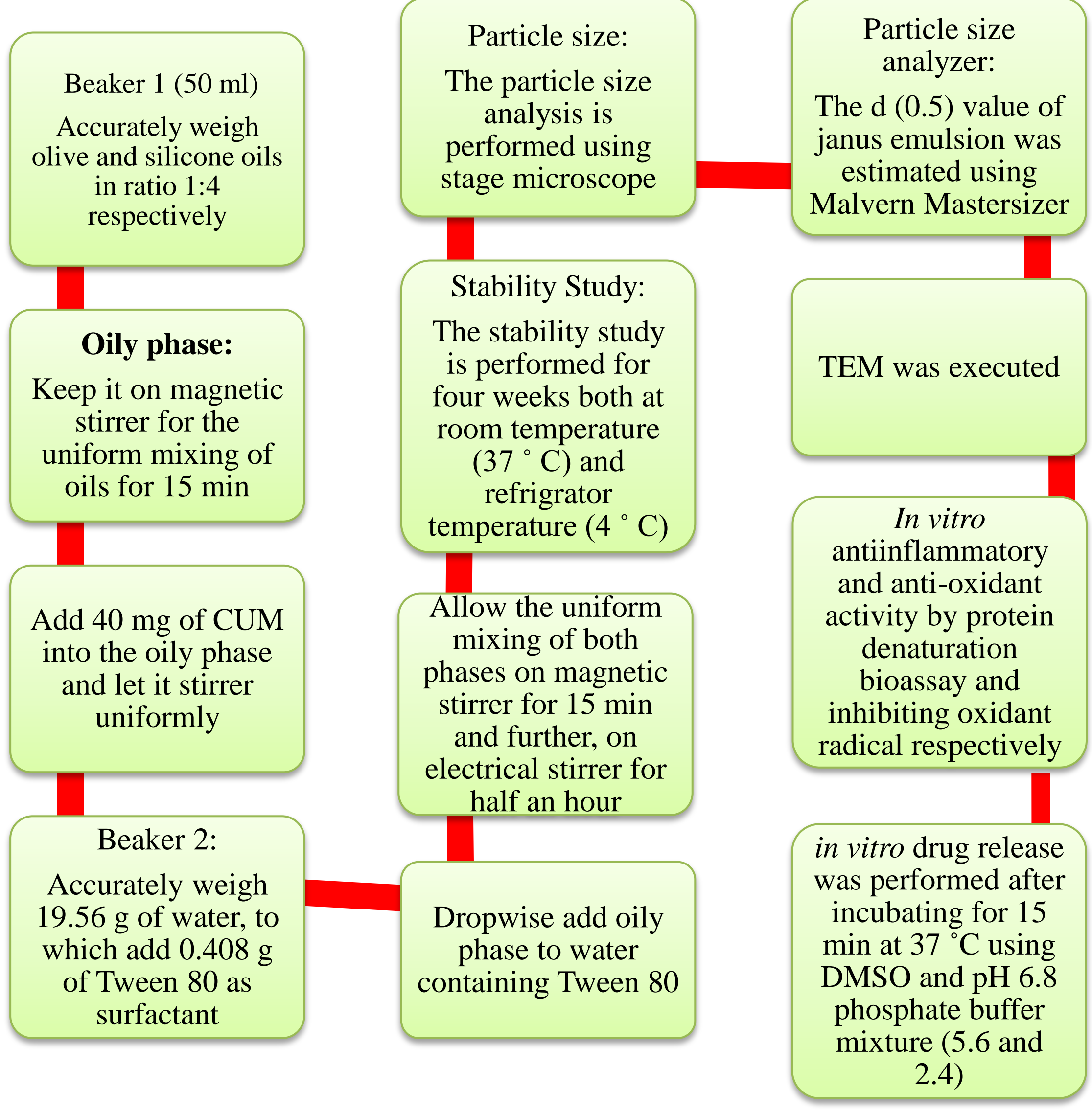


Fig 6.7: Steps involved in the preparation of Janus emulsion

Flow chart 1: The steps involved to prepare Janus emulsion using laboratory scale method:



6.2.12 Methods for evaluation

6.2.12.1 Determination of particle size by microscopy

a) Microscope was cleaned properly and was placed in proper place where light was suitable for projection.

b) Then the eye-piece was fixed in microscope with micrometre.

c) The eye-piece micrometre was calibrated with a standard stage micrometre.

d) Then sample was mounted on the plain side.

e) Then the size of particles was measured with the help of eye-piece micrometre.

f) 100 particles were counted accurately and then picture was captured through Asus Zenfone.

6.2.12.2 Determination of particle size by Malvern Mastersizer

Mastersizer equipment was developed by Malvern, instrumentation limited; London, UK was used for determining the mean particle size of the emulsion. This equipment works on the basis of Frauenhofer or Mei scattering theory. About 100-200 µl of emulsion was mixed with 150 ml of dispersion water (Hydro S). A laser beam of He-Ne light source was used to detect the particle size ranging from 0.02-2000 microns.

6.2.12.3 Transmission electron microscopy (TEM)

6.2.12.3.1 Preparation of staining solution

a) Add 0.1 g of sodium phosphotungstate (PTA) powder to 10 ml of distilled water in a shell vial. Check the PTA solution pH with the pH meter and adjust the pH to 7.2-7.4 with the 0.1 N NaOH solutions.

b) Add 500 µg bacitracin powders to the phosphotungstic acid solution.

6.2.12.3.2 Staining Procedure

a) Pick up a carbon – coated grid with forceps and push the forceps locking ring down so that the grid is held firmly. Lay forceps down on petridish lid with tips extending over the edge with the grid held coated-side up. The low thermal stability of the carbon-coated pioloform film on copper grids (piloform grids) in combination with PTA for contrast in the TEM is utilized.

b) The prepared CUM-loaded coarse emulsion was diluted at 5 times with deionized water. A drop of the diluted emulsion was applied to a carbon-coated copper grid using Pasteur pipette.

c) After 3-5 min, remove diluted emulsion by touching ragged torn edge of filter paper to the edge of the forceps jaws (where they contact the grid) until the grid surface is nearly dry. Never let the grid surface totally dry out because it will produce a coating of culture or body fluids and yield an excessively dirty grid.

d) Add one drop of PTA/bacitracin solution to the grid.

e) After 1 min, dry the grid as before with the ragged torn edge of filter paper except this time; dry the grid quickly and completely. Immediately touch the sample-side of the grid to a clean piece of filter paper in the bottom of a petridish. Slide a fresh piece of filter paper down between the forceps jaws to push the dried grid out of the forceps tips and onto a clean, dry part of the filter paper in the petridish.

f) Let the grid dry for 15 min in the petridish (covered) and then examine with a TEM.

6.2.12.4 Thin layer chromatography (TLC)

- **Mobile phase:** Chloroform and methanol in ratio of 9.5:0.5
- **Stationary phase:** Silica gel G
- **Visualising aid:** UV rays (Gupta et. al., 2011)

Procedure

To perform the TLC, the mobile phase containing chloroform and methanol in the ratio of 9.5:0.5 was kept in kept in a TLC chamber and closed with lid for a period of 40 to 50 minutes to make the chamber saturated with mobile phase vapours. Slurry (5-10% w/v) of silica gel G was made in distilled water and poured onto a glass plate/slide to make the stationary phase. The prepared plate was activated by keeping it in a hot air oven for a period of 40 minutes at 100° C. A methanolic solution of an accurately weighed quantity of CUM and equivalent amount of processed and unprocessed CUM in Janus emulsion were applied using a micropipette directly on the TLC plate, leaving 2 cm

from the border. The plate is developed with the prepared mobile phase mixture in a TLC chamber for half an hour until the solvent rise up to $3/4^{th}$ of the plate. After development, the plate was dried at room temperature to make the plate ready for visualization. The identification of spot was done with fluorescent indicator UV254 and UV366. The R_f value was calculated with help of the following equation, and the experiment was repeated thrice under the identical experimental conditions.

$$\textbf{Retention factor (Rf)} = \frac{\textbf{Distance travelled by sample}}{\textbf{Distance travelled by solvent}} \qquad \textbf{eq. (3)}$$

6.2.12.3 Drug amount at oil water interface

The EE of the emulsions were determined by measuring the concentration of CUM in the aqueous layer obtained by ultracentrifugation (UC) (Wang et al., 2006). Centrifugation was carried out using a HITACHI ultracentrifugation apparatus, operated at 12,000 rpm at 25° C for 15 min. Polyallomer tubes were used and their bottoms were pricked after centrifugation with a syringe needle to collect the aqueous phase. Concentrations of CUM in both the aqueous layer and the whole emulsion were determined by UV spectrophotometer.

The % EE was calculated according to the following equation (Groves et al., 1985; Ferezou et al., 1994):

$$\textbf{\% Drug Entrapment Efficiency} = \frac{(\text{C total} * \text{V total}) - (\text{C water} * \text{V water})}{(\text{C total} * \text{V total})} \textbf{ X 100} \qquad \textbf{eq. (4)}$$

Where,

- C_{total} is total drug concentration (40 mg)
- V_{total} is total volume of emulsion (25 ml)
- C_{water} is drug concentration in water phase

$$\textbf{Drug amount on oil} - \textbf{water interface} = \frac{\textbf{Drug amount in whole emulsion – drug amount in water phase}}{\textbf{initial amount of the drug added}} \textbf{ X 100} \qquad \textbf{eq. (5)}$$

1.2.12.6 *In vitro* drug release

- **a)** The CUM-loaded Janus emulsion was prepared freshly.
- **b)** Three 10 mL capacity beakers were taken and washed properly with double distilled water (DDW).
- **c)** All three beakers were kept in hot air oven at 60 ± 5 ˚C for drying.
- **d)** The 1.6, 0.8 and 0.2 mL of pure-CUM loaded Janus emulsion were diluted in 8 ml of DMSO and pH 6.8 phosphate buffer solution in ratio (7:3) to elicit 1:5, 1:10 and 1:40 dilution ratios, respectively.
- **e)** The samples were placed in glass vials and incubate at 37 ˚C on water bath throughout the experiment.
- **f)** 0.4 mL of sample was withdrawn from each glass vial at a time interval of 1, 16, 36, 86,110 and 180 minutes and no replenishment was made at each of the sampling times.
- **g)** All the withdrawn samples (0.4 mL) were diluted separately up to 5 mL using DMSO and pH 6.8 phosphate buffer mixtures (7:3).
- **h)** The absorbance of all the samples was taken at 558 nm.

6.2.12.7 Antioxidant activity

6.2.12.7.1 Preparation of DPPH methanolic solution

0.1 mM solution of DPPH was prepared in ethanol and 0.5 ml of this solution was added to 1.5 ml of CUM solution in ethanol at different concentrations (15-45 µg/ml). Further, votex these solutions thoroughly and incubate in dark for 30 min. after half an hour, the absorbance was measured at 517 nm against the blank lacking scavenger. The standard curve was prepared using different concentrations of DPPH solutions.

6.2.12.7.2 Preparation of standard sample solution

Accurately weigh 5 mg of the ascorbic acid, dissolve it in 1 ml of methanol and further add 6 ml of DPPH methanolic solution at different concentrations (15-45 µg/ml). This will give a standard solution with light yellow appearance.

6.2.12.7.3 Preparation of control sample solution

Accurately weigh 5 ml of prepared emulsion, dissolve it in 1 ml of methanol and further, add 6 ml of DPPH methanolic solution. This will give a standard solution with orange color appearance.

6.2.12.7.4 Preparation of test solution

Accurately weigh 5ml of the three respective emulsions (emulsion containing pure CUM, emulsion containing modified vapour pressure processed CUM and emulsion containing Rota evaporated processed CUM) in 1 ml methanol to which add 6 ml of DPPH solution at different concentrations (15-45 µg/ml).

Note: Lower absorbance at 517 nm represents higher DPPH scavenging activity. The % DPPH radical scavenging activity of sample was calculated from the decrease in absorbance at 517 nm DPPH radical's concentration. The anti-oxidant activity that is % free radical inhibition was calculated using the formula using eq. (6).

$$\% \textbf{ Free radical inhibition} = \frac{Ac - As}{Ac} X\, 100 \qquad \textbf{eq. (6)}$$

Where,

- Ac = absorbance of control (all ingredients except drug)
- As = Absorbance of emulsion containing drug

6.2.12.8 Antiinflammatory activity

a) In brief, the CUM (40 mg) - loaded coarse emulsion (25 ml) was prepared freshly.

b) From this, 1 ml of emulsion (equivalent to 1.6 mg of CUM) was taken and diluted with 100 ml phosphate buffer solution pH 7.4.

c) From this stock solution, 2 ml (equivalent to 0.032 mg of CUM) was mixed with 0.2 mg of egg albumin powder and 2.8 ml of phosphate buffer solution of pH 7.4. Hence the final concentration was 6.4 µg/ml.

d) Step (2) was repeated by using 2, 3, 4 and 5ml of emulsion (equivalent to 3.2, 4.8, 6.4 and 8.0 mg of CUM respectively).

e) The step (3) was repeated by taking 2 ml from each of the solutions obtained from step (4) therefore, the obtained final concentration was 128, 192, 256 and 320 μg respectively. Similarly, the final solution concentration corresponding to step (4) were 25.6. 38.4, 51.2 and 64 μg/ml.

f) A control solution was also prepared using emulsion without drug and phosphate buffer solution of pH 7.4 and step (2) and (3) were followed.

g) All the samples were incubated at 37± 2˚C in an incubator for 15 minutes.

h) After incubation, all the samples were heated for 70˚C for 5 min in a water bath.

i) After cooling to room temperature, the absorbance of all the solutions were measured blank.

j) The reference standard stock solutions were prepared by dissolving 40 mg of diclofenac sodium in 13 ml of phosphate buffer solution of pH 7.4. Steps (2) and (3) were performed to get the final concentration of 24.8. 37.2, 49.6 and 62 μg/ml and the absorbance values for these solutions were also measured at 660 nm.

k) The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\% \ \mathbf{inhibition} = \mathbf{100}\left(\frac{\mathbf{Vt}}{\mathbf{Vc}} - \mathbf{1}\right) \qquad \textbf{eq. (7)}$$

6.2.12.9 Stability of emulsion

The stability of the Janus emulsion was carried for the four weeks at different temperatures. The Janus emulsion stability was carried out at 37 ˚C (room temperature) and 25 ˚C (refrigerator). The stability of the Janus emulsion was estimated microscopically with the appearance of Janus particles.

6.2.12.10 Method to find potential toxicity (Haemolysis test)

RBC breakdown assay

a) Freshly collected blood from a healthy male volunteer was mixed with anticoagulant solution (74.8 mm sodium citrate/ 38.07 mm citric acid/ 124.3 mm glucose 1:1:19 v/v) and centrifuged at 650 mg for 10 min.

b) The supernatant was discarded and the erythrocytes were re-suspended in phosphate buffered saline (PBS, pH=7.4). Next, the erythrocytes were washed three times with an isotonic buffer (PBS), and upper phase with a buffy coat

containing precipitated debris and serum proteins was carefully removed at each wash step.

c) After last washing, the packed cells were suspended in a buffer to a haematocrit of 50%.

d) All the erythrocytes suspensions used in the experiments were prepared daily.

e) To determine the haemolytic effect, 100 μL of each samples (processed and unprocessed drug powder particles and Janus emulsions containing equivalent amount of processed and unprocessed drug) containing 83, 136 and 144 μg of CUM (calculated from normalized drug entrapment efficiency values of 46.83 %, 75.63 % and 80.03 % observed respectively with the Janus emulsion containing pure CUM, Janus emulsion containing modified vapour pressure processed CUM and Janus emulsion containing anti-solvent precipitated processed CUM) were diluted with 10 ml of buffer and 1 ml from this diluted samples (containing 8.3, 13.6 and 14.4 μg of CUM) was added to 20 μL of erythrocytes suspension 950% haematocrit) and adjusted to a 4-ml volume with PBS.

f) The samples were stirred and incubated for 30 mins at 37 ˚C. Debris and intact erythrocytes were removed by centrifugation at 650xg for 10 min.

g) The haemoglobin released into the supernatant was detected spectrophotometrically at 490 nm against the corresponding blank sample.

h) The haemolytic effect, measured as the percentage of haemolysis (H), was determined on the basis of released haemoglobin, according to the following formula.

$$\mathbf{H}\ (\%) = \frac{\mathbf{As - Ac1}}{\mathbf{Ac2 - Ac1}}\ \mathbf{X\ 100} \qquad \textbf{eq. (8)}$$

Where,

H = Haemolysis

As = Absorbance of the sample (CUM and CUM-loaded emulsion)

Ac1 = Absorbance due to mechanical haemolysis (RBC 20 μL + 4 mL PBS)

Ac2 = 100% haemolysis (RBC 20 μL + 4 ml DDW)

i) The procedure from (e) to (h) was repeated with emulsion prepared based on Olive oil only (without silicone oil) and also without CUM.