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Highlights

- Determinants of Hypertension
- Venous Thromboembolic Events
- Evaluation of Methanolic Extract
- Preliminary Phytochemical Analysis

Discovering Thoughts, Inventing Future

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Neuromodulation of Mu-opioid Receptor (MOR-1) Gene (OPRM1) Alternatively-Spliced Variants Following Exposure to Morphine with Alma Fig (*Ficus carica*) Leaf Extract in Human Neuroblastoma (SH-SY5Y) Cells: Review & Pilot Study

By Alrena V. Lightbourn, Zhi-Ping Zhu & Carl B. Goodman

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Abstract- The role of morphine in regulating the mu-opioid receptor (MOR-1) relative to pain is well-established. Efforts are ongoing to elucidate the pharmacological significance of newly identified MOR-1 alternative splice variants. Aberrant splicing events have been implicated in a growing number of diseases, including cancer, but it is uncertain whether any pharmacological benefit may be derived from the use of these variants. Chronic use of opioids yields tolerance, withdrawal, and potentially fatal addiction. With current interests so high on developing marijuana as a marketable drug, there is concern whether its introduction as a mainstay may interfere with pain medications, such as opioids, for which there is a growing concern of epidemic proportions. We, therefore, hypothesized that the introduction of traditional herbal medicines while taking morphine would interfere with normal pain receptor functions.

Keywords: Ficus carica; G-protein; mu-opioid receptor; opioid; morphine; tolerance; alternative splicing; alternative splice variant; differentiation; posttranscriptional; gene expression; Nanodrop; vehicle; agonist; antagonist; neuropeptide; cultivar.

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Neuromodulation of Mu-opioid Receptor (MOR-1) Gene (OPRM1) Alternatively-Spliced Variants Following Exposure to Morphine with Alma Fig (*Ficus carica*) Leaf Extract in Human Neuroblastoma (SH-SY5Y) Cells: Review & Pilot Study

Alma Fig Effect on MOR-1 Variant mRNA

Alrena V. Lightbourn [α], Zhi-Ping Zhu [σ] & Carl B. Goodman [ρ]

Abstract- The role of morphine in regulating the mu-opioid receptor (MOR-1) relative to pain is well-established. Efforts are ongoing to elucidate the pharmacological significance of newly identified MOR-1 alternative splice variants. Aberrant splicing events have been implicated in a growing number of diseases, including cancer, but it is uncertain whether any pharmacological benefit may be derived from the use of these variants. Chronic use of opioids yields tolerance, withdrawal, and potentially fatal addiction. With current interests so high on developing marijuana as a marketable drug, there is concern whether its introduction as a mainstay may interfere with pain medications, such as opioids, for which there is a growing concern of epidemic proportions. We, therefore, hypothesized that the introduction of traditional herbal medicines while taking morphine would interfere with normal pain receptor functions. We tested this hypothesis by chronically (48hrs) exposing human neuroblastoma (SH-SY5Y) cells to a pain medication (morphine) followed by a natural herb, and measuring its effect on the expression of MOR-1 alternatively-spliced variants. (RA)-differentiated human neuroblastoma (SH-SY5Y) cells treated with morphine (10 μM), fig leaf extract (3 μL/30 mL media), or both for 48 hours, were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) using the Bio-Rad iCycler/MyiQ™. Of the seven fig (*Ficus carica* L.) cultivars (Green Ishia, Brown Turkey, Mission, Alma, Giant Celeste, Nero, Hollier) identified for this pilot study, Alma fig leaf extract was selected for combined therapy with morphine. Statistically significant differential regulation of MOR-1 alternative splice variants was widely observed in control, morphine, Alma fig leaf extract, and morphine/Alma fig samples. The results of this pilot study confirm our hypothesis that MOR-1 splice variants are differentially regulated following chronic exposure to morphine and *Ficus carica*. Further examination of the relationship between morphine and herbs used in traditional medicine may enhance our understanding of the mechanistic basis of morphine tolerance and may give clues concerning the therapeutic benefit of using *Ficus carica* leaf extracts to counteract the effects of opioids via targeted posttranscriptional isoforms of the mu-opioid receptor. (333 words)

Keywords: Ficus carica; G-protein; mu-opioid receptor; opioid; morphine; tolerance; alternative splicing; alternative splice variant; differentiation; posttranscriptional; gene expression; Nanodrop; vehicle; agonist; antagonist; neuropeptide; cultivar.

Abbreviations

GPCR: g-protein-coupled receptor;
OPRM1: mu-opioid receptor gene;
MOR-1: mu-opioid receptor subtype 1;
ASV: alternatively-spliced variant;
qRT-PCR: quantitative reverse-transcription polymerase chain reaction;
mRNA: messenger ribonucleic acid;
DNA: deoxyribonucleic acid;
SH-SY5Y: human neuroblastoma cells;
RA: trans-retinoic acid;
cDNA: DNA complementary to RNA;
BACT: beta-actin;
MOR-1: mu-opioid receptor subtype 1;
MOR-1A: mu-opioid receptor subtype 1, alternatively-spliced variant A;
MOR-1B1: mu-opioid receptor subtype 1, alternatively-spliced variant B1;
MOR-1B2: mu-opioid receptor subtype 1, alternatively-spliced variant B2;
MOR-1B3: mu-opioid receptor subtype 1, alternatively-spliced variant B3;
MOR-1B4: mu-opioid receptor subtype 1, alternatively-spliced variant B4;
MOR-1B5: mu-opioid receptor subtype 1, alternatively spliced variant B5;
MOR-1K1: mu-opioid receptor subtype 1, alternatively-spliced variant K1.

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I. Introduction

The human nervous system is composed of a complex and highly organized network of excitable tissues, neurons, and their receptors, effectors, interneurons, neurotransmitters, hormones, and a host of structures through which to orchestrate anatomic homeostasis, in tandem with the endocrine system. The neuron is the central functional unit of the nervous system tasked with synchronizing action potentials that govern sensory, integrative, and motor functions. This small but rapid and highly efficient communication system regulates processes of learning and memory, sensations (e.g., pain, thermal, tactile, proprioceptive), perception, analgesia, differentiation, development, emotional responses, emotional behaviors, wakefulness and sleep (Tortora & Grabowski, 2003; Massirer et al., 2010). An extensive annual review (not elaborated here) covering the endogenous opioid system reflects its diverse contributions to matters concerning: "behavior, pain, and analgesia; stress and social status; tolerance and dependence; learning and memory; eating and drinking; alcohol and drugs of abuse; sexual activity and hormones; pregnancy; development and endocrinology; mental illness and mood; seizures and neurologic disorders; electrical-related activity, neurophysiology and transmitter release; general activity and locomotion; gastrointestinal, renal, and hepatic function; cardiovascular responses; respiration and thermoregulation; [and] immunological responses" (Bodnar & Klein, 2006). The activities of each body system are regulated through action potentials generated by the neuron. Extensive alternative splicing in the nervous system is, therefore, likely to play a role in many of these physiological processes and conditions (Grabowski & Black, 2001).

The endogenous opioid system, which is resident within the mammalian nervous system, plays a significant role in a variety of physiological processes within the mammalian body. The nervous system is naturally resilient. It does not easily succumb to toxicity or insult, instituting neuroadaptive changes and self-recovery instead. Importantly, the nervous system has a unique integrative capacity to resolve at the molecular level problems that arise at the cellular level. Psychotropic substances of plant origin, such as morphine from the opium poppy (*Papaver somniferum*), mimic the action of neurotransmitter action of enkephalins, the natural ligand for this receptor. Specialized (sensory) neurons throughout the body mediate pain sensation by regulating the human mu-opioid receptor (MOR-1) gene (OPRM1) expression. Among natural medicines used for pain, the fig (*Ficus carica*) plant is commonly not listed. It more often finds prominence relative to conditions such as diabetes, hyperlipidemia, eczema, psoriasis, constipation, skin tumors and warts, and vitiligo (Jellin et al., 2009), some of which are side effects of opioids (Stephan & Parsa, 2016). Hence, it would be interesting to learn of a role for the fig plant in the mediation of pain, analgesia, and opioid receptor pharmacology.

Consumption of Pain Medications

Pain drugs are the second most dominant pharmaceutical class in the global market. US market. For centuries, the alkaloid-derived morphine (Figure 1) has remained the prototypical anti-nociceptive agent (WHO, 1986; Pasternak, 2001; Vanquelin & von Mentzer, 2007; Yu & Sadee, 1988; Corbett et al., 2006). Its analgesic superiority underscores the use of morphine as a preferred clinical and non-medical psychotherapeutic drug (Tremblay & Hamet, 2010). Contrary to controversial reports that the United States alone utilized eighty percent (80%) of the global supply of morphine (Manchikanti et al., 2006, 2010; CNBC, 2016), recent findings of the International Narcotics Control Board (INCB) scale this gross overestimate down to just 30.2%. In 2010, with only 5.2% of the world's population, the United States (US) led the world in its consumption of morphine, averaging roughly 55.4% (22.9 tons) of the world's morphine (UN, 2011). Unfortunately, this figure rose to 57.3% in 2013 (GCDP, 2015) but later dropped to 29.3% by 2015 (Chris, 2018). Of the morphine manufactured globally in 2011, the United States produced 78.4 tons, or 19% (UN, 2011). In 2018, the United Nations INCB reports flagged the US with having a continuing disparate consumption of opioid analgesics (UN, 2018).

Cause for Concern: Variability of Response to Drug-Disease Interactions

The observation that there exist inter- and intra-individual differences in response to prescribed or illicitly used medications reinforces the significance of modern-day precision medicine (Samer et al., 2006; Rollason et al., 2008; Dorn & Cresci, 2008). Characteristically, differences in age as well as in drug interactions with cytochrome P450 metabolic enzymes have historically separated subpopulations from generalized use of medications to more patient-centered determinations of appropriate pharmacological treatments (Samer et al., 2006; Rollason et al., 2008; Finklestein, 2017; Krebs & Milani, 2019). The ongoing discussion of genetic polymorphisms continues to inform this process.

The current literature on alternative splicing (Figure 2) indicates that this posttranscriptional process is essential for life but may to contribute inter- and intra-individual variability by altering gene function (House & Lynch, 2008); switching substrate specificity (Christmas et al., 2001; Bauman et al., 2009); or causing disease (e.g., cancer) through aberrant splicing events (Faustino 2003; Buratti et al., 2006). At least ten alternatively-spliced isoforms (Figure 3) of the human mu-opioid receptor (MOR-1) gene (OPRM1) have been identified (Pasternak and Pan, 2009). Moreover, each splice

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variant may exhibit different agonist-induced activation, signal transduction, and protein expression patterns. Within pharmacogenomics, understanding how a person's genetic profile influences his response to a drug is a treasured clinical endeavor, in which is embedded great hope for the improvement in the medical use and administration of drugs across all ages and stages of development (Finklestein, 2017). Central to these efforts is the drug-receptor (Danhof et al., 2007; Ploeger et al., 2009).

Historical Receptor Theories

The receptor is the smallest pharmacological unit necessary to differentiate between drugs (Kenakin, 2004). The idea that receptors are responsible for drug effects is an evolving theory that developed between the late 19th century and early 20th century due to the pioneering work of several scientists. Credited for the concept of "locus of effect," Claude Bernard (1813-1878) pioneered a methodological blueprint for elucidating the specificity and selectivity of drug action (Bernard, 1856). As an offshoot of interest in finding a more rational approach for therapy, Hungarian scientist Rudolf Buchheim (1820-1879) opened the first pharmacology laboratory with the intent of measuring drug effects and their associated mechanisms of action (Hollinger, 1997). In1848, Blake framed the structure-activity relationship (SAR). He made observations to correlate the biological effects of a substance with its chemical structure, arguing that a specific component was responsible for the observed change rather than the complex as a whole. He garnered theoretical support from the later work of Arrhenius on electrolytic dissociation, and Crum Brown and Fraser who found differing physiological actions by alternating alkaloid structures. Hans Horst Meyer (1899) and Charles Ernest Overton (1901), independently described lipid solubility. Among other discoveries during this period, other scientists were discovering the high physiological specificity of opioids on smooth muscle versus smooth muscles.

The existence of receptors was first suggested in 1878 by John Newport Langley (1852-1925), followed in 1905 by him coining the term "receptive substances." Paul Ehrlich (1854-1915) specifically introduced the concept word "receptor" in his medical correspondence wherein he attributed a therapeutic effect only to an agent having "the right sort of affinity" (Ehrlich, 1923; Hollinger, 1997). Ehrlich envisioned receptors as "side-chains" that interacted with a "combining group of the protoplasmic molecule to which the introduced group is anchored" in mammalian cells (Hollinger, 1997).

As he studied the interactions between enzymes and substrates, Emil Fischer, a German chemist, and enzymologist, was the first to propose a "lock and key" relationship between a drug and its receptor. Fischer postulated that a specific similar geometric configuration of the receptor was necessary for a chemical reaction to proceed from contact between these molecules. The precise fit was required to produce the optimal response (Hollinger, 1997). This theory was consistent with existing science showing that the primary amino acid sequence of a protein determines its three-dimensional structure, and according to Christian Anfinsen, these molecules were capable of unfolding (denaturing) and folding (renaturing) to vary their conformation (Hollinger, 1997).

As Alfred Joseph Clark (1885-1941) first proposed, the "receptor occupancy theory" demonstrates the interaction of a first messenger (e.g., a signal molecule such as a drug, chemical, or neurotransmitter) with its specific physiological cellular receptor (e.g., mu-opioid receptor, subtype 1 – MOR-1) to produce a measurable biological response (Limbird, 2005). The curve of a dose-response graph resembles a mathematical hyperbole. Subsequent research by Raymond P. Ahlquist (1914-1983) led to the discovery of unique differences between alpha- and beta-adrenoceptors, and this served one catalyst for Sir James Black's (1988) Nobel winning interrogation of drugs with receptor-selective subtypes. Not too long afterward, Gilman and Rodbell won the Nobel Prize for GPCRs and receptor coupling. These monumental works have moved the field of receptor pharmacology to uncharted heights that continue to influence today's society. A summary of the general characteristics of receptors appears in Table 3.

Characteristics of the Mu-opioid Receptor

The opioid receptor is a member of Class A of the superfamily of guanosine nucleotide-binding protein (G-protein)-coupled receptors (GPCRs) that constitute ~3% of the human genome. They contain a total of 7 extracellular and intracellular transmembrane (7TM) domains linked to three subunits: alpha – α, beta – β, and gamma – γ. The beta and gamma subunits are tightly linked, while the alpha subunit more freely associates or dissociates from this dimer. Ligands approach and engage the receptor from the extracellular space, and receptor activation results in coupling to heterotrimeric G-proteins on the intracellular face of the membrane. Binding and hydrolysis of guanosine triphosphate (GTP) to the α-subunit of the G-protein activates a resting receptor and results in the dissociation of the βγ subunit from the receptor. The βγ subunit in conjunction with downstream effectors or the GTP-bound α-subunit can trigger a plethora of downstream events. The association of guanosine diphosphate (GDP) with the α-subunit promotes its further association with the βγ subunits, returning it to an inactive state. Opioid receptor signals are transduced by intracellular inhibitory G-proteins (G_i/G_0), which are relatively resistant to tolerance and desensitization (Pasternak, 2001; Pan et al., 1999, 2001, 2005).

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In studies conducted by Kuhar (2010), autoradiographic localization of opiate receptors rendered these microscopic molecules as being saturable, primarily particulate-bound, and accessible in proportion to the high level of activity of opiate drugs, but seemingly unaffected by drugs not of opiate origin. Observed drug effects are a consequence of physiological responses associated with control mechanisms that permit access to the drug via the action of its physiological intermediate.

When an endogenous opioid, such as enkephalin, binds to and activates MOR-1, the ensuing conformational changes help to modulate synaptic transmission in the neuron, ultimately resulting in a cascade of intracellular signaling events that amplify the signal and produce a diverse array of pharmacological outcomes, depending on the tissue (Limbird, 2005; Benye et al., 2015). The intensity of the response to a signaling 'messenger' molecule (ligand) depends in large part on the specificity with which that ligand attaches to the receptor recognition site (binding pocket).

MOR-1 Alternative Splicing

Although the pharmacological and physiological attributes of morphine and its receptors have been extensively elaborated over the past three decades (Zadina et al., 1993; Pasternak, 2001; Kuhar, 2010; Benyhe et al., 2015), recent identification of multiple splice variants of the mu-opioid receptor (MOR-1) (Zadina et al., 1993; Pan et al., 1999; Pan et al., 2001; Braaco & Kearney, 2003; Pan et al., 2005; Oldfield et al., 2008; Shabalina et al., 2009) raises a plethora of questions as to the functional significance of these variants (Pasternak, 2001). Gene expression is regulated at the transcriptional level; hence, contributions by MOR-1 splice variants are of interest. There is also a dearth of information about mechanisms that account for the substantial diminution of the efficacy of morphine, which gives rise to the development of tolerance following longterm use (Yu & Sadee, 1988; Zadina et al., 1993; Taylor & Fleming, 2001; Willner et al., 2014).

Up to sixty percent of the human genome is estimated to contain alternatively-spliced gene isoforms (Lee & Irizarry, 2003). Aberrant splicing events have been implicated in a growing number of diseases, including cancer (Mercadante & Kole, 2000; Braaco & Kearney, 2003; Lee & Irizarry, 2003; Brinkman, 2004). The C-terminus of cell-surface, seven-transmembranes (7TM) receptors is home to the biggest array of splice variants, which may occur at more than one site on the receptor, adding to the complex structure of the gene (Kilpatrick et al., 1999). The opioid receptor is one example of a 7TM receptor within the guanine nucleotide-binding proteins (g-protein)-coupled receptor (GPCR) family, which transfers signals for hundreds of cellular receptors. This highly diversified family of g-protein receptors execute neurotransmission, cellular differentiation, hormonal activities, signal transduction, metabolism, and other processes (Kilpatrick et al., 1999). The pharmacological significance of newly identified mu-opioid receptor (MOR-1) alternative splice variants (Pasternak & Pan, 2004; House & Lynch, 2008) has not been characterized relative to drug response mechanisms and may inform the issue of morphine tolerance. Among the 70-90% of cancer patients requiring individualized opioid therapy for intense chronic pain, the response to prototypical opiates like morphine is highly variable, necessitating dose escalation with an increased risk of developing tolerance (WHO, 1986; Bracco and Kearsey, 2003).

Given the central role of MOR-1 in pain mediation, brain reward systems, opiate addiction and homeostasis (Cox, 1991; Trujillo & Akil, 1991; Di Chara & North, 1992; Meunier, 1992; Law & Loh, 1999; Nestler & Aghajanian, 2007), a plethora of questions exist as to the functionality of these alternatively spliced variants, selectivity of ligand binding, and the implications of these potential associations in disease and therapy (Braaco & Kearney, 2003; Lee & Irizarry, 2003; Brinkman, 2004). The functional capacity of MOR1 splice variants is unknown, and it is yet unclear whether alternatively-spliced isoforms respond to botanical products like the prototypical ligand for the mu-opioid receptor, morphine.

Discovery of the Medicinal Properties of Morphine

Recognition of the pharmacological properties of plants and the medicinal use of morphine date far back to ancient civilizations (e.g., Sumeria, Egypt, Ancient Greece, Roman Empire). Among modern narcotic analgesics, morphine is the oldest and remains the gold standard (prototype) that is the most widely used. Morphine is the principal active ingredient in the opium poppy (*Papaver somniferum)*. The groundbreaking discovery of morphine as the first alkaloid isolated from naturally occurring plant species by Wilhelm Serturner, a German Pharmacist, forever changed organic chemistry, medicine, and history. Notwithstanding, morphine is also present in appreciable amounts in Theriaca, laudanum, Doveri, and paregoric (Benyhe et al., 2015). The recreational use of opium is widely (but not exclusively) practiced in the Middle East and the Far East provinces (e.g., Arabia, Turkey, Iran, India, and China), but the illicit sale and use of opium and its synthetic derivatives have since reached global proportions (Benyhe et al., 2015).

Subsequent determination of the chemical formula of morphine (Laurent, 1847), the structure of morphine (Robinson, 1925), and its industrial extraction (Kabay, 1925) have led to the total synthesis of morphine (1952-1956) (Gates and Tsudi, 1952-1956). Gulland elucidated the stereochemical structure of morphine as having a rigid phenanthrene ring system

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comprised of five condensed rings (A – phenolic, aromatic; B – cyclohexane; C – cyclohexanol, cyclohexene; D– N-methyl-piperidine, piperidine; and E – a partially saturated furan ring, tetrahydrofuran) (Gulland and Robinson, 1925). The phenolic makeup of the A-ring makes it a weak acid (Lemke, 2003). Primary and secondary alcohol (-OH) group substitutions at the A-ring C3 and the D-ring C6 positions, respectively, confer chemical reactivity on the molecule. Morphine has five chiral centers at carbon-5 (C5), C6 C9, C13, and C14 positions. The piperidine constituency of the D ring renders morphine the classification of a weak base (Benyhe et al., 2015). By this latter classification, morphine "does not readily donate its electrons and forms an unstable ammonium ion that dissociates readily with a large dissociation constant (K_a), and thus has a small pK_a" (Lemke, 2003).

Brief History of the Fig Plant

The fig tree is a deciduous shrub of the Moraceae family that can typically grow to massive proportions up to 15 to 30 feet tall (and often wide) (Patil & Patil 2011). These keystone plant species favor tropical and subtropical regions where it is sunny, and the soil is well-drained, and are capable of withstanding drought conditions (Jeong & Lachance, 2001; Solomon et al., 2006; Ahmed et al., 2012; Mawa et al., 2013). The fig tree is one of the oldest known plants in history, dating as far back as the days of Creation as penned in the Bible (Saif et al., 2020). Its fruit is the botanical embodiment of stem tissue, called a syconium, which contains both male and female flower parts (Aradhya et al., 2010). Hence, these plants develop partheno-carpically without pollination (Flaishman et al., 2008; Mawa et al., 2013; Lyons & McEachern, 2019). Fig trees in the Arabian Peninsula date as afar back as 3000 BC (Saif et al., 2020). They were first cultivated approximately 11,000 years ago, presumably as far east as South-Central Asia before spreading westward (Ashton, 2019) toward Turkey, Syria, and the Mediterranean basin (Saif et al., 2020), soon becoming a favorite of Greek, Egyptian and Roman civilizations (Saif et al., 2020).

Transport of the duly named "Mission" fig plant by Spanish missionaries to the West in the mid-nineteenth century accounts for its arrival in Texas and California (Aradhya et al., 2010). The various classes of horticulturally-important, edible figs (i.e., Capri, San Pedro, Smyrna, Common Fig) are so classified based on the floral biology and pollination behavior (Aradhya et al., 2010). Of these, the Common Fig (*Ficus carica*) genus is the only one that persistently yields fruit. It produces over 2000 varieties of fruit and semi-tropical plants, and over 700 varieties of common garden figs (Aradhya et al., 2010; Ashton, 2019).

Herb-Disease Interactions

The therapeutic efficacy of *Ficus carica* has also not been demonstrated; neither is it established as having potential drug-herb interactions with narcotic drugs or nutrients (Jellin et al., 2009). Yet, fig is a highly abundant staple of the Mediterranean diet (Croft, 1998, Visioli & Galli, 2001; Solomon et al., 2006; Gomez-Romero et al., 2007) and its global health-promoting potential is underutilized (Dragsted et al., 1993; Genkinger et al., 2004; Solomon et al., 2006).

Many of the pharmacological effects associated with the fig plant (Patil & Patil, 2011) correlate to its high antioxidant capacity (Sirisha et al., 2010). The leaves possess a robust antioxidant potential and the highest concentration of phenolics (Sirisha et al., 2010). In traditional applications, both the fig leaves and the fruits are beneficial "as laxative, stimulant, against throat diseases, antitussive, emollient, emmenagogue, and resolvent" (Bellakhdar et al., 1991; Guarrera et al., 2003; Konyahoglu et al., 2005). The high flavonoids content of fig leaves (Saeed & Sabit, 2002) is of interest given the tremendous anti-inflammatory, cardioprotection, and anti-cancer effects associated with this phytochemical (Croft; 1998; Jeong & Lachance, 2001; Amir et al., 2007). Reports (Jouad et al., 2001; Leoporatti & Ivancheva, 2003; Konyahoglu et al., 2005; Jellin et al., 2009) have also upheld the unique hypoglycemic properties of fig leaves in humans (Serraclara et al., 1998) and rats (Perez et al., 1996).

Studies assessing the α-tocopherol, flavonoid, and phenol contents relative to the antioxidant activity of fig leaves have established the antioxidant capacity of *Ficus carica* leaf extracts and raised hopes for the role of α-tocopherol in clarifying its mechanism of action (Konyahoglu et al., 2005). Phytosterols have, in part, been credited for the hypocholesterolemic effect observed in Mission fig (Jeong & Lachance, 2001). In Ghana, the *Ficus* plant is a popular galactagogue (Bekoe et al., 2018). Also, the nutritive value of the high dietary fiber and high mineral content of figs is superior to many other fruits. There is an established high correlation between total polyphenols, or total anthocyanins, and the antioxidant capacity of Mission fig, and to a much lesser extent, Brown Turkey fig class (Solomon et al., 2006; Crisosto et al., 2010). The potential irritant effects of *Ficus carica* leaves on the ears of albino mice (Saeed & Sabir, 2002) is presumably due to the psoralens present in leaves (Jellin et al., 2009), but their standard use in the dietary prevention of anemia and as an anti-helminthic underscores the benefit of their medicinal properties (Saeed & Sabir, 2002; Jeong et al., 2009). Thus, similar to fruit, the value of commonly discarded fig (*Ficus carica*) leaves for health promotion and medical treatment offers potentially viable opportunities for further research and the discovery of nutraceuticals and pharmacotherapies from promising cultivars.

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Herb-Drug Interactions

Individual *Ficus carica* cultivars reportedly vary in the antioxidant capacity (Crisosto et al., 2010; Mawa et al., 2013) and the antioxidant and phenolic constituencies are may be an artifact of the genetic makeup (Scalzo et al., 2005; Aradhya et al., 2010). Among commercial varieties (e.g., Mission, Brown-Turkey, Bursa, Brunswick, and Kadota), Mission fig has the highest concentration of total polyphenols, total flavonoids, total anthocyanins, and Trolox equivalent antioxidant capacity (TEAC) in fig fruits, skins and pulps (Solomon et al., 2006). Another study by Jeong and colleagues (2009) also found Mission fig to have exceptional total polyphenol content in the leaf extract compared to that of Brown Turkey. This advantage may be tied to the original geographical and/or geological source of the Mission fig tree, brought to the US by Franciscan missionaries; it may be less resistant to genetic changes imposed by environmental differences.

Researchers have examined the weight-of-evidence of herb-drug interactions pertaining to the fig leaf and have concluded that concern is both relevant and valid based on available literature (i.e., non-randomized clinical trial [RCT]; non-quantitative systematic review; lower quality RCT; clinical cohort study; case-control study; historical control; or epidemiologic study) (Jellin et al., 2009). The severity of interactions of fig leaf with two drugs has been rated as "moderate," and caution is advised with these combinations. Clinical research or pharmacokinetic data in humans suggests that this interaction is "probable," meaning that it will occur in a significant portion of patients. Fig leaf may interact with anti-diabetic drugs or insulin. In both cases, fig leaf lowers blood glucose levels by enhancing the effect of hypoglycemic drugs (Jellin et al., 2009). Mechanistically, fig leaf is capable of improving glucose update by skeletal muscle (Jellin et al., 2009). However, there was no clear evidence as to the implications of other herb-drug interactions [such as motor function drugs (e.g., skeletal muscle relaxants – benzodiazepines; anti-seizure drugs - phenobarbital), or centrally acting drugs that affect smooth muscles (e.g., morphine)] or herb-disease interactions [such as drugs affecting skeletal muscle tone in Parkinson's Disease and other movement disorders].

Finally, an analysis of the mineral content of the fructus and folium of *Ficus carica* L. revealed superior concentrations of calcium, potassium, magnesium, phosphorus and sulfur in folium (27,611 ± 152 μg/g; 16,000 ± 234 μg/g; 3,565 ± 174 μg/g; 1,285 ± 31 μg/g; and 1,150 ± 67 μg/g respectively) versus fructus (6,006 ± 613 μg/g; 13,892 ± 415 μg/g; 1,381 ± 186 μg/g; 1,054 ± 44 μg/g; and 536.1 ± 7.5 μg/g respectively) (Ficsor et al., 2013). No documented fig-food interactions, or morphine-mineral interactions, were found (Jellin et al., 2009). It is also unclear whether high concentrations of the above minerals in fig folium or fructus affect the bioavailability of morphine. Hence, caution to comply with existing recommended dietary reference intake (DRI) values (*calcium*: RDA 1,000mg/d –male, 1,200 mg/d – female; *potassium*: DRI 4.7 g/d; *magnesium*: RDA 420mg/d –male, 320 mg/d – female; *phosphorus*: RDA 700mg/d, Upper Limit 4,000 mg/d) (NAS, 1997, 2001, 2005, 2011) is important. Comparatively, opportunities to remove nutritional deficiencies through supplemental use of *Ficus carica* may become necessary. These attributes support the assumption that the leaves may also possess a high nutritional and medicinal value that warrants further exploration. Nine cultivars grown in the United States were selected for further interrogation in the present research (Table 1).

PCR Comes of Age

One celebrated outcome of the Human Genome Project is its propulsion of the field of molecular genomics into the research spotlight. Innovations in molecular biology and pharmacogenomics, as well as technological advances in quantitative real-time polymerase chain reaction (qRT-PCR), have led to the identification of several new human mu-opioid receptor (MOR-1) splice variants that to date have not been fully characterized (Saiki et al., 1985, 1988; Watson, 1990; Olson, 1993; Collins et al., 1998; Pollock, 2002). The polymerase chain reaction (PCR) is a sensitive technology which was discovered by Kary Mullis in 1983 for the original purpose of improving DNA quantification (Mullis et al., 1986; Bartlett & Starling, 2003). However, PCR has also led to our improved knowledge of biological processes such as RNA transcription, cellular growth, and proliferation, differentiation, development Advances in PCR technology have advanced the field of gene expression analysis for over twenty-five years, namely: the introduction of real-time PCR (Williams, 2009), discovery of reverse transcription (Baltimore, 1970; Temlin & Mizutani, 1970); qRT-PCR and the emergence of sophisticated instrumentation to detect vanishingly small quantities of nucleic acids (Saiki et al., 1985, 1988; Zimmerman & Mannhetter, 1996; Snider et al., 2001; VanGuilder et al., 2008).

PCR capitalizes on the well-established significance of DNA in living cells following elucidation of the genetic code (Watson & Crick, 1953) as well as the central dogma of molecular biology which posits that the uni-directional flow of genetic information is from DNA to RNA, via transcription, and from mRNA (the product of transcription) to protein, via translation (Crick, 1958). Transcription is the first and rate-limiting step in the process of gene expression. The term 'gene expression' is synonymous with 'messenger ribonucleic acid (mRNA) levels.' Quantitative real-time polymerase chain reaction precisely and reliably measures gene expression levels of specific nucleic acid sequences

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(Kaltenboeck & Wang, 2005; Bustin, 2000, 2010; Bustin & Nolan, 2004; Bustin et al., 2005). Close examination of emerging patterns of gene expression can provide insight into physiological responses to cellular stressors or signals, or whether the genes are functionally related (Pollock, 2002). The evident superiority of qRT-PCR surpasses older technologies (e.g., Northern blot, RNase protection assays) and affirms its designation as the "gold standard" or method-of-choice for analyzing gene expression of modest numbers of genes (Nedelman, 1992).

The biological significance of qRT-PCR to modern biology and biomedical sciences is irrefutable. In the aftermath of discoveries made in the Human Genome Project, scientists have begun to explore more intensely the molecular underpinnings of sickness, chronic disease, and drug interactions in the body (Snider et al., 2001; Bernard & Wittwer, 2002; Pollock, 2002; Kaltenboeck & Wang, 2005). Answers to elusive medical conditions, such as cancer and drug tolerance, can be elucidated at the molecular level to shed greater insight into the nature of these conditions as well as the mechanisms by which they occur (Braaco & Kearney, 2003; Brinkman, 2004; Kaltenboeck & Wang, 2005). The use of this generalized PCR equipment to characterize the plant genome is not new. The efficiency of quantitative real-time PCR in detecting posttranscriptional changes in human cells induced by natural plant products gives way to future consideration of the mechanistic actions of plant extracts, as well as drug-herb interactions. The enhanced capacity for comparative analysis of critical neurological systems, such as the opioid system, using this technology, as well as the potential discovery of relevant interventions to eliminate the cause of chronic diseases, gives hope for the future of medicine.

Using Ancient Medicinal Herbs to Counteract an Age-Old Enigma

For centuries, morphine has been utilized as the prototypical analgesic drug in the treatment of chronic and intractable pain. Morphine exerts its pain-relieving effects primarily through the mu-opioid receptor (MOR-1). Moreover, ancient civilizations have used the fig (*Ficus carica* L.) plant for wound healing, digestive clearance, and as a hypolipidemic agent in diabetes. Since morphine induces constipation and hyperglycemia, we hypothesized that fig leaf extract could attenuate or abrogate these adrenergic effects of morphine, as well as its central effects.

With the recent advances of receptor polymorphisms and gene splicing, several variant forms of MOR-1 have recently been identified. Through the use of polymerase chain reaction (PCR) technology, the fields of molecular genetics and pharmacology have begun to converge and so enable a deeper understanding of the mechanistic basis of opioid-related diseases, like opioid dependence, addiction, and withdrawal, which are chronic outcomes of morphine tolerance.

The present study examined the prototypic effects of morphine on MOR-1 variant mRNA expression compared that of fig (*Ficus carica*) leaf extract. The objective of this study was to employ advanced molecular genetics techniques, including real-time qRT-PCR, to assess the ability of fig leaf extract, in the presence or absence of morphine, to interact with MOR-1 receptor alternatively-spliced variants (ASVs) and to modify its transcriptional machinery, a rate-limiting step in MOR-1 protein synthesis and functionality of the mu-opioid receptor (OPRM1).

II. Materials and Methods

a) General Chemical Reagents and Pharmacologic Agents

Isopropanol, chloroform, morphine, distilled water, and consumable supplies were supplied by Sigma-Aldrich (St. Louis, MO, USA). Absolute ethanol (100%) was obtained in-house.

i. *qRT-PCR Chemicals*

The DNAse treatment and removal kit were purchased from Ambion (Foster City, CA, USA). iQ™ SYBR Green Supermix and iScript cDNA Synthesis System were ordered from Bio-Rad Laboratories (Hercules, CA).

ii. *Ficus carica L. Cultivars*

"Just Fruits & Exotics Nursery" of Crawfordville, FL donated the *Ficus carica* leaves from nine cultivars (Green Ishia [FIG1], Brown Turkey [FIG2], Mission [FIG3], Alma [FIG4], Celeste [FIG5], Giant Celeste [FIG6], Black Jack [FIG7], Nero [FIG8] and Hollier [FIG9]). In this paper, we present a pilot study of only one of these cultivars, the Alma fig, as well as some common characteristics of the other fig varieties and their extracts (Table 1).

b) Methods

i. *Human Neuroblastoma (SH-SY5Y) Cell Line*

Human neuroblastoma (SH-SY5Y) cells are epithelial cells that were derived from the bone marrow of a metastasized tumor originating in the brain of a 4-year old girl. SH-SY5Y cells are stable neuroblasts that were thrice-cloned from the original SK-N-SH cell line (Ross et al., 1983). The expression of mu-opioid receptors in SK-N-SH cells was determined to be five times higher than that of delta-opioid receptors (Yu et al., 1986), which is reproduced in SH-SY5Y subclones (Yu et al., 1986). SH-SY5Y cells are a reproducible cell model for studying the biochemical correlates of opiate efficacy and tolerance (Yu and Sadee, 1988). Additionally, SH-SY5Y cells can express several distinct phenotypes, including immature neuroblast forms that differentiate into mature neurons (Ross et al., 1983)

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following treatment with retinoic acid (RA) (Pahlman et al., 1984). It is advantageous to use an *in vitro* model of specialized nervous system cells (i.e., neurons) in this study because isolation of the effects of chemicals at the molecular level is complicated by the heterogeneity of *in vivo* nervous system networks within tissues.

ii. *Cell Culture*

Human neuroblastoma (SH-SY5Y) cells (ATCC, Bethesda, MD) were maintained under sterile conditions in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 (1:1) (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Sigma, St. Louis, MO). The cells were stored in a humidified incubator at 37°C and 5% CO_2. Cells were grown to 70-80% confluence (15 x 10^6 cells) before differentiation with retinoic acid (RA).

a. *RA Differentiation of SH-SY5Y Cells*

Human neuroblastoma SH-SY5Y cells were the first neuronally-derived cell line suitable for studying chronic opiate (morphine) effects (Zadina et al., 1993). Two unique properties of SH-SY5Y cells that auger well for their use in opioid research are their constitutive expression of the mu opiate receptor in measurable quantities (Toll, 1990; Bare et al., 1994; Edsjo et al., 2007) and the rare ability of this cell line to be induced to express the neuronal phenotype by addition of retinoic acid (Sidell 1982; Sidell et al., 1983; Zadina et al., 1993; Yu and Sadeee, 1988; Borner et al., 2007). Differentiation of SH-SY5Y cells ensued after addition of *all-trans* retinoic acid (10 mM), dissolved in absolute ethanol, to fresh culture medium (final concentration: 10μM). At 70-80% confluence (~15 x 10^6 cells), the cells were exposed to RA for 48 hrs before all media was removed and refreshed. The cells were reintroduced to RA (10μM) for a further 24-hr period.

b. *Treatment of SH-SY5Y Cells*

Three categories of treatments were used in the experiments, including morphine (the prototypical, full opioid receptor agonist and potent analgesic), fig leaf extract (a crude botanical product), or control (media alone). Plated cells, at or near confluence, were randomly assigned to the respective treatment groups.

A stock solution of morphine (10 mM) was prepared according to manufacturer's instructions. SH-SY5Y cells were treated with morphine (10μM), fig leaf extract (3μL/30μL media), or both for 48 hours as independent triplicate samples. On harvesting, the SH-SY5Y cells were thrice washed with 1X PBS then stored at -70°C until analysis.

c. *Differentiated but Untreated SH-SY5Y Cells [Experimental Controls]*

The measurement outcome (dependent variable) of these experiments is "gene expression." The independent variables examined in this study are "treatment," "time," and "genotype." Control (media alone) received no chemical treatment but a supplemental volume of media only. Triplicate control plates accompanied each set of independent experiments. We evaluated MOR-1, MOR1-A, MOR1-B1, MOR1-B2, MOR1-B3, MOR-1B4, MOR1-B5, MOR1-K1, and β-ACT genotypes (n=8) in each sample at one of three time-points (n=3), 24hr, 48hr, and 72hr. Plated cells were randomly allocated to the respective treatment groups at or near confluency. A total of 9 plates comprising a single experimental unit were analyzed.

d. *Phase Contrast Microscopy*

We monitored the growth of SH-SY5Y cell cultures differentiated with RA using phase-contrast microscopy to ensure the progression of neuritogenesis under experimental conditions.

c) Primer Design

Primer pairs (Table 2) were designed to recognize and amplify the specific region within the c-terminus of the MOR-1 gene where the variant is located. Invitrogen's OligoPerfect™ Designer online system was used to design forward and reverse oligonucleotide primers (Invitrogen, Carlsbad, CA), no more than 26 base pairs in length. Primers to detect the expression of transcription factors are listed in Table 3.

d) RNA Isolation

All procedures were performed according to the manufacturer's protocols. Trizol extraction (Invitrogen, Carlsbad, CA) and DNase treatment of total ribonucleic acid (RNA) were used to purify the sample for reverse transcription (Turbo DNA-free™ Kit, Ambion, Foster City, CA). The final concentration of the reaction mixture was 10 μg of RNA/50 μl DNase cocktail. Total RNA concentrations were measured before proceeding with the remaining procedures. Samples with Nanodrop™ A_{260}/A_{280} absorbance ratios≥1.8 and adequate RNA concentrations were selected for amplification by real-time quantitative polymerase chain reaction (qRT-PCR) using the Bio-Rad iCycler/MyIQ™ (Bio-Rad, Hercules, CA).

e) cDNA Synthesis

First-strand cDNA was reverse-transcribed from purified RNA using a 20 μl reaction mixture (iScript™ cDNA Synthesis Kit, Bio-Rad, Hercules, CA) containing 5 μl (1 μg) of RNA. Samples were incubated in the thermocycler for 30 minutes (25°C, 5 min; 42°C, 15 min, twice; 85°C, 5 min) before storage at -70°C.

f) Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR)

Reverse-transcribed cDNA was amplified by RT-qPCR using iQ SYBR Green Supermix® ™ (Bio-Rad, Hercules, CA) with human forward/reverse primer-probe sets for β-actin (housekeeping gene), human MOR1A (HMOR-1A), HMOR-1B1, HMOR-1B2, HMOR-1B3, HMOR-1B4, HMOR-1B5 and HMOR-1Y genes (25 μl

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SYBR, 20 μl water, 3 μl primer, 2 μl cDNA). Optimization of the thermal profile at 95°C (5 min) was followed by a 2-step amplification and melt process over 40 cycles (95°C, 10 sec; 55°C, 45 sec). The thermal cycler (Figure 4) was set to proceed at 95°C (1 hr) followed by 55°C (1 hr), and finally, 55°C (10 sec). The specificity of qRT-PCR was checked by examining melt curves generated for each set of triplicate control, treated, and standard curve samples.

i. *Standard Curve*

Relative gene expression levels were determined using the standard curve method. For each primer pair (forward and reverse), the amplification efficiency for each gene of interest was based on a four-point, 5-fold sample dilution series. Signal threshold cycle, or C_t, values were logarithmically transformed to extrapolate the level of MOR-1 variant mRNA relative to β-actin (reference gene). Relative expression of an individual gene of interest was defined as the percentage ratio of log-transformed C_t values for treated (C_t-treat) samples to the C_t value for β-actin, relative to controls (C_t-control).

g) Quality Assurance/Control

The specificity of qRT-PCR was checked by examining melt curves generated for each set of triplicate control, treated, and standard curve samples. Before each use, the Nanodrop™ and analytical scale were sanitized and calibrated between after each use according to standard laboratory procedures. Microvolumes of each sample were loaded onto the pedestal as RNA purity and quantity were assessed spectrophotometrically.

h) Normalization of qRT-PCR Data

Replicate samples should be run at least in triplicate assays, and the experiments repeated at least thrice. Relative gene expression is calculated based on the standard curve method (as above) and normalized by housekeeping and control genes. The data should be subjected to dual normalization based on the ratio of 'log base two' equivalent values for target and control genes. For example, the proportion of 'target gene: housekeeping gene' and 'target gene: control gene' were computed.

i) Statistical Analysis

The measurement outcome (dependent variable) of these experiments is "gene expression," quantified as relative messenger RNA (mRNA) levels. The independent variables examined in this study are "treatment" and "genotype." For qRT-PCR, MOR-1 and selected variant forms (i.e., MOR-1A, MOR-1B1, MOR-1B2, MOR-1B3, MOR-1B4, MOR-1B5, and MOR-1K1), as well as β-ACT genotypes (n=8) were evaluated.

The data (mean ± SEM) represent triplicate assays of samples obtained from three independent experiments. The small sample size represents a limitation on this pilot study that does not appear to deface the quality of the data. Dataset organization and basic descriptive statistics were calculated using Microsoft Excel®. The data were then normalized to β-actin mRNA and control values.

Statistical analyses and graphics were performed using the Prism 6.0™ software. Statistical significance of t-tests was set at an alpha level of $p<0.05$.

III. Results

a) RA Differentiation in SH-SY5Y Cells

Retinoic acid (RA) induced differentiation of native SH-SY5Y cells into cells morphologically classifiable as neuronal cells, as confirmed by the presence of dendritic formations, neurite outgrowths, and axonal extensions.

i. *Expression of MOR-1 ASVs in Experimental Control SH-SY5Y Cells*

Untreated but RA-differentiated (control) cells exhibited significant ($p<.0001$) constitutive, differential expression of all MOR-1 alternative splice variants as well as beta-actin (Figure 5). MOR-1B4 was undetected.

b) Tolerogenic Effect of Morphine on Differentiated SH-SY5Y Cells

Based upon our present preliminary screen of mRNA extracted from RA-differentiated human neuroblastoma (SH-SY5Y) cells and analyzed by qRT-PCR using Bio-Rad Thermocycler/MyIQ® software, prototypical opioids induced measurable tolerogenic effects within 48 hours of opioid exposure. Treatment with morphine alone significantly down-regulated MOR-1B1 (77.32%, $p<.0001$), MOR-1B2 (70.10%, $p<.0001$), MOR-1B3 (92.96%, $p<.005$), and MOR-1K1 (82.18%, $p<.0001$) mRNA levels relative to controls in BACT-normalized samples. In contrast, MOR-1A (179.7%, $p<.05$) and MOR-1B5 (109.3%, $p<.0001$) in these samples were significantly up-regulated following morphine treatment (Figure 6). Compared to the responses of the other variants in morphine-treated samples, the effect on MOR-1A may be an outlier as an artifact of a small sample size. MOR-1B4 was undetected.

c) Effect of Fig Leaf Extract on Differentiated SH-SY5Y Cells

Treatment of SH-SY5Y cells with Alma fig leaf extract for 48hr substantially amplified the expression of MOR-1A (396.1%, $p<.0001$), MOR-1B1 (440.1%, $p<.05$), MOR-1B2 (239.1%, $p<.05$), MOR-1B5 (259.1%, $p<.005$), and MOR-1K1 (230.2%, $p<.05$), relative to controls. There was inadequate evidence of MOR-1B3 down-regulation by the Alma fig cultivar (Figure 7).

Compared to the responses of the other variants in Alma fig leaf extract-treated samples, the effect on MOR-1B3 may be an outlier as an artifact of a

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small sample size. MOR-1B4 was undetected. On examining patterns of expression following administration of Alma fig only, the inflated mRNA values suggest a synergistic interaction with endogenous opiates.

d) Combined Effect of Morphine and Fig Leaf Extract on MOR-1 ASV Expression

Cells initially treated with morphine were subsequently treated with Alma fig leaf extract. In the morphine/Alma fig treatment group, MOR-1B1 (459.6%, $p<.05$), MOR-1B2 (228.1%, $p<.05$), MOR-1B5 (301.8%, $p=.0069$), and MOR-1K1 (156.6%, $p<.005$) mRNA levels were found to be up-regulated, whereas MOR-1A1 (65.4%, $p>.05$) and MOR-1B3 (77.02%, $p<.0001$) mRNA levels were down-regulated (Figure 8).

Compared to the responses of the other variants in morphine-treated samples, the effect on MOR-1A and MOR-1B3 may be an outlier as an artifact of a small sample size. MOR-1B4 was undetected.

The addition of Alma fig extract completely abrogated the tolerogenic effects of morphine on MOR-1B1, MOR-1B2, and MOR-1K1. When morphine was administered alone, there was an observed characteristic attenuation of mRNA levels. The marked inflation of mRNA levels in morphine/fig samples suggests that Alma fig leaf extract may indeed have "inverse agonist" properties, as it is customary for inverse agonists to elicit the opposite effect to that of an agonist to the receptor. This pattern of opposites was observed relative to MOR-1A, MOR-1B1, MOR-1B2, and MOR-1K1 when comparing "morphine"-treated to "morphine/fig"-treated samples. Also prominent were the double to triple amplification of MOR-1B5 signals, approximating additive effects (morphine alone – 109.3%; Alma fig alone – 259.10%; morphine+Alma fig – 301.8%).

IV. Discussion and Conclusion

a) Model Selection

Human neuroblastoma (SH-SY5Y) cells were the first neuronally-derived cell line deemed suitable for the *in vitro* study of chronic opiate (morphine) effects (Zadina et al., 1993). SH-SY5Y cells also continue to be a reliable model for its current use in the expression of mu-opioid receptor variants (Toll, 1990; Bare et al., 1994; Edsjo et al., 2007) due to its high constitutive expression of this receptor and its ability to be induced by retinoic acid to express the neuronal phenotype (Sidell et al., 1983; Zadina et al., 1993; Yu and Sadee, 1988).

b) Pilot Study

This study confirms our hypothesis that mu-opioid receptor (MOR-1) alternatively spliced variants are sensitive and differentially responsive to prototypical opioids as well as botanical products (i.e., *Ficus carica* leaf extract). Relative to ligand binding, these data indirectly suggest that some constituent in the fig (*Ficus carica*) leaf appears compatible with the mu-opioid receptor, can bind to the MOR-1 binding site, and is capable of triggering a signaling cascade that elicits genetic effects at successive DNA, RNA and posttranscriptional levels. This constituent is probably structurally similar to morphine or one of its precursors. Given the dependency of gene expression on the tightly regulated, successive steps of transcription, it is reasonable to conclude that there is evidence for functional modulation of MOR-1 in neurons.

c) Limitations

Due to the small sample size of this pilot study, expanded analyses under experimental conditions are warranted. The data confirm the efficacy of custom-designed primers for targeting specific regions of the OPRM1 gene and the distinctive value of individual *Ficus* cultivars in interacting with the mu-opioid receptor.

d) Conclusions

Alma fig leaf extract targets specific exons within the mu-opioid receptor (MOR-1) gene (OPRM1) to reverse morphine-induced down-regulation of MOR-1 alternatively-spliced variants. The differential expression of MOR-1 isoforms in response to Alma fig and/or morphine/Alma fig leaf extract, as well as the appearance of additive, synergistic and inverse interactions between these botanicals and human cells, suggests a potential future role in resolving inter- and intra-individual differences in response to morphine. The current finding brings us a little closer to an approach for discriminating the functions of individual MOR-1 ASVs and may play a future role in identifying herb-drug interactions that affect medical prescribing and medication management practices.

Further work is needed to characterize Alma fig leaf extract and its implications for cancer and pain therapy. Added attention to the reversing effects of Alma fig leaf extract following morphine treatment is needed as this outcome may prove useful for reversing morphine-induced side effects, such as constipation and tolerance

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Conflict of Interest

The author knows of no financial interest or any conflict of interest relative to this article.

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Figure Legends

Figure 1: Pain relief: "The Prototypical Role of Morphine in Solving a Universal Health Condition".

Figure 2: Splicing versus alternative splicing in the multistage process of eukaryotic gene expression. Splicing occurs co-transcriptionally prior to the export of mRNA from the nucleus to the cytosol. After transcription and RNA processing, the mature mRNA has an open reading frame (ORF) that encodes a human mu-opioid receptor protein of 400 amino acids.

Figure 3: Schematic representation of the role of alternative splicing in mu-opioid receptor (MOR-1) physiology.

Figure 4: Some instruments of gene expression analysis. The process of gene expression analysis typically involves four (4) biochemical steps: RNA isolation, removal of contaminating DNA, first-strand complementary DNA (cDNA) synthesis, and real-time reverse transcription (RT)-PCR, in that order. A homogenizer, Nanodrop™, and thermocycler are relevant and necessary to complete this process.

Figure 5: Relative expression of MOR-1 variant mRNA in untreated, retinoic acid (RA)-differentiated (Control) human neuroblastoma (SH-SY5Y) cells. Human neuroblastoma (SH-SY5Y) cells were grown under sterile conditions in phenol red-free, Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 (1:1) containing 2.5 mM L-glutamine and supplemented with 10% fetal bovine serum (FBS, v/v) and 100 Units of penicillin/0.1 mg streptomycin. The cells were maintained in a humidified incubator at 37°C, 95% O_2/5% CO_2. At roughly 80% confluence, SH-SY5Y cells were treated with retinoic acid (RA, 10 μM) for 72 hr then harvested for RNA isolation. A reaction mixture containing 10 μg of RNA/50 μl DNase cocktail was used in conjunction with the iScript™ cDNA Synthesis Kit to prepare first-strand cDNA for qRT-PCR. Target nucleic acid sequences of MOR-1 variants (i.e., MOR-1A, MOR-1B1, MOR-1B2, MOR-1B3 and MOR-1B5) were simultaneously amplified and quantified via qRT-PCR. Data are mean ± SEM of $\log_2$ transformed C_T values based on the variant-specific standard curve. Statistical significance presented as: ***, $p<.0001$.

Figure 6: Effect of morphine on mu-opioid receptor (MOR-1) alternatively-spliced variants expression in retinoic acid (RA)-differentiated human neuroblastoma (SH-SY5Y) cells. Human neuroblastoma (SH-SY5Y) cells were grown under sterile conditions in phenol red-free, Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 (1:1) containing 2.5 mM L-glutamine and supplemented with 10% fetal bovine serum (FBS, v/v) and 100 Units of penicillin/0.1 mg streptomycin. The cells were maintained in a humidified incubator at 37°C, 95% O_2/5% CO_2. At roughly 80% confluence, SH-SY5Y cells were treated with retinoic acid (RA, 10 μM) for 72 hr, then with morphine (10 μM) for 48 hr before being harvested for RNA isolation. A reaction mixture containing 10 μg of RNA/50 μl DNase cocktail was used in conjunction with the iScript™ cDNA Synthesis Kit to prepare first-strand cDNA for qRT-PCR. Target nucleic acid sequences of MOR-1 variants (i.e., MOR-1A, MOR-1B1, MOR-1B2, MOR-1B3 and MOR-1B5) were simultaneously amplified and quantified via qRT-PCR. Data are mean ± SEM of $\log_2$ transformed C_T values based on the variant-specific standard curve. Data manipulations were performed in Microsoft Excel and the graph was developed in GraphPad Prism Version 6.0. Statistical significance presented as: *, $p<.05$; **, $p<.005$, ***, $p<.0001$.

Figure 7: Effect of of Alma Fig (*Ficus carica*) leaf extract on mu-opioid receptor (MOR-1) alternatively-spliced variants expression in retinoic acid (RA)-differentiated human neuroblastoma (SH-SY5Y) cells. Human neuroblastoma (SH-SY5Y) cells were grown under sterile conditions in phenol red-free, Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 (1:1) containing 2.5 mM L-glutamine and supplemented with 10% fetal bovine serum (FBS, v/v) and 100 Units of penicillin/0.1 mg streptomycin. The cells were maintained in a humidified incubator at 37°C, 95% O_2/5% CO_2. At roughly 80% confluence, SH-SY5Y cells were treated with retinoic acid (RA, 10 μM) for 72 hr, then treated with Alma Fig leaf extract (3μL/30μL media, v/v) before being harvested for RNA isolation. A reaction mixture containing 10 μg of RNA/50 μl DNase cocktail was used in conjunction with the iScript™ cDNA Synthesis Kit to prepare first-strand cDNA for qRT-PCR. Target nucleic acid sequences of MOR-1 variants (i.e., MOR-1A, MOR-1B1, MOR-1B2, MOR-1B3 and MOR-1B5) were simultaneously amplified and quantified via qRT-PCR. Data are mean ± SEM of $\log_2$ transformed C_T values based on the variant-specific standard curve. Statistical significance presented as: *, $p<.05$, **, $p<.005$; ***, $p<.0001$, $p=.0009$.

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Figure 8: Combined effect of morphine plus "Alma" fig leaf extract on the expression of mu-opioid receptor (MOR-1) alternatively-spliced variants in human neuroblastoma (SH-SY5Y) cells. Expression of mu-opioid receptor (MOR-1) alternatively-spliced variant (MOR-1; MOR-1A; MOR-1B1; MOR-1B2; MOR-1B3; MOR-1B5; MOR-1K1) genes in morphine-treated human neuroblastoma (SH-SY5Y) cells, as a percent of control values and normalized to beta-acting (housekeeping gene). Bars represent mean ± SEM. Data manipulations were performed in Microsoft Excel and the graph was developed in GraphPad Prism Version 6.0. Statistical significance presented as: *, $p<0.05$; ***, $p<0.0001$, $p=.0004$, $p=.0009$.

Figures

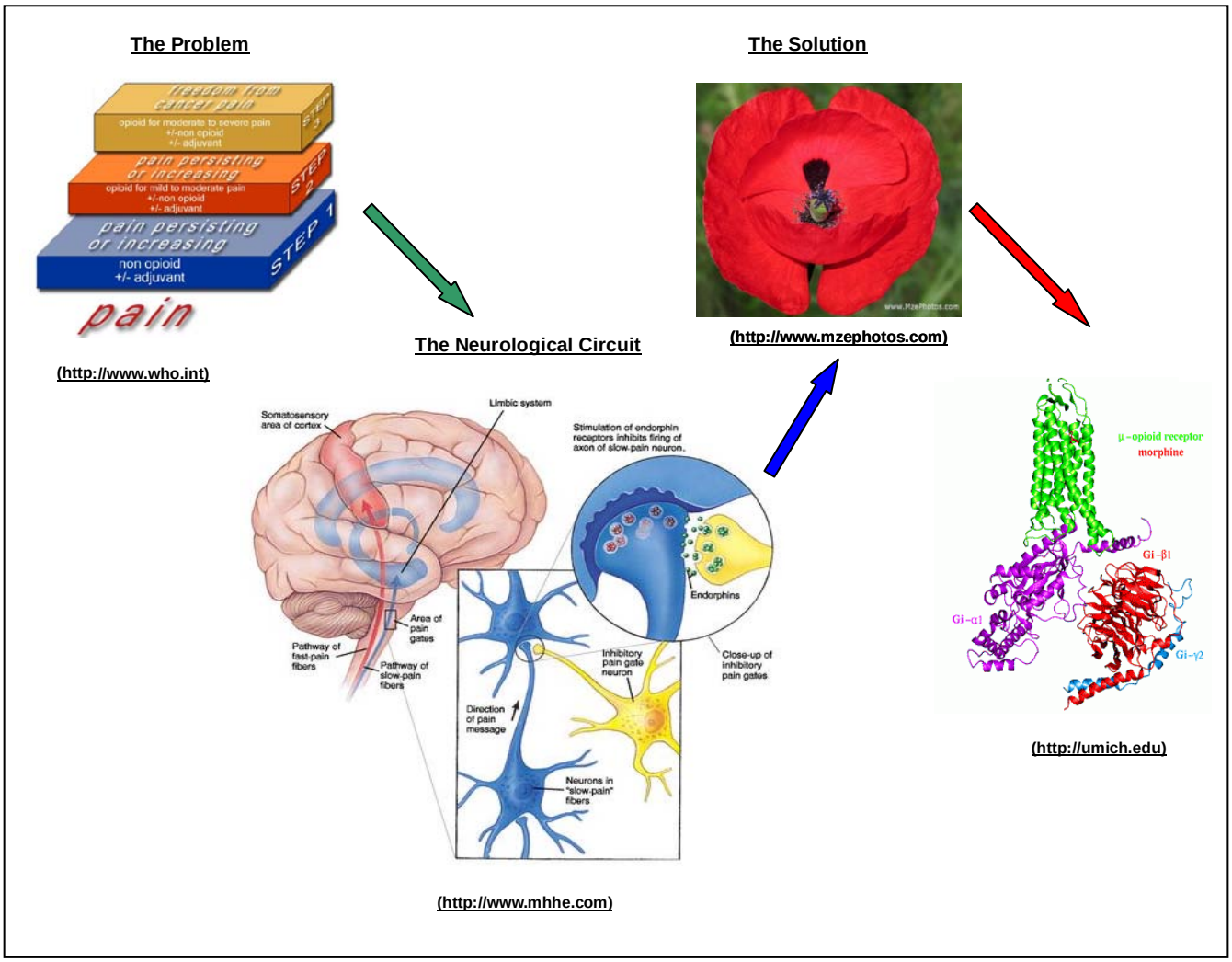
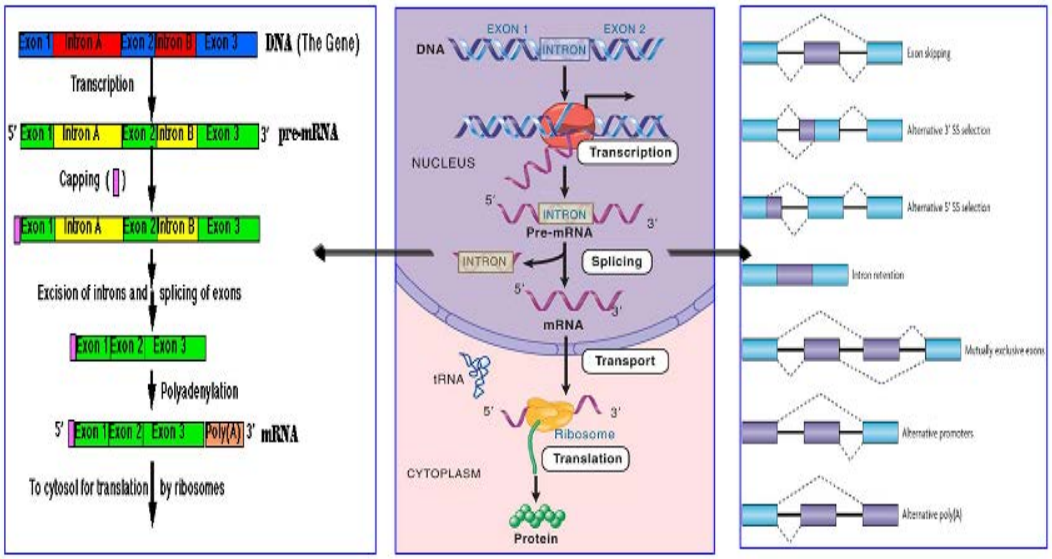


Figure 1: Pain Relief: "The Prototypical Role of Morphine in Solving a Universal Health Condition"

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(http://www.google.com/images)

Figure 2: Splicing versus. Alternative Splicing in the Multistage Process of Eukaryotic Gene Expression

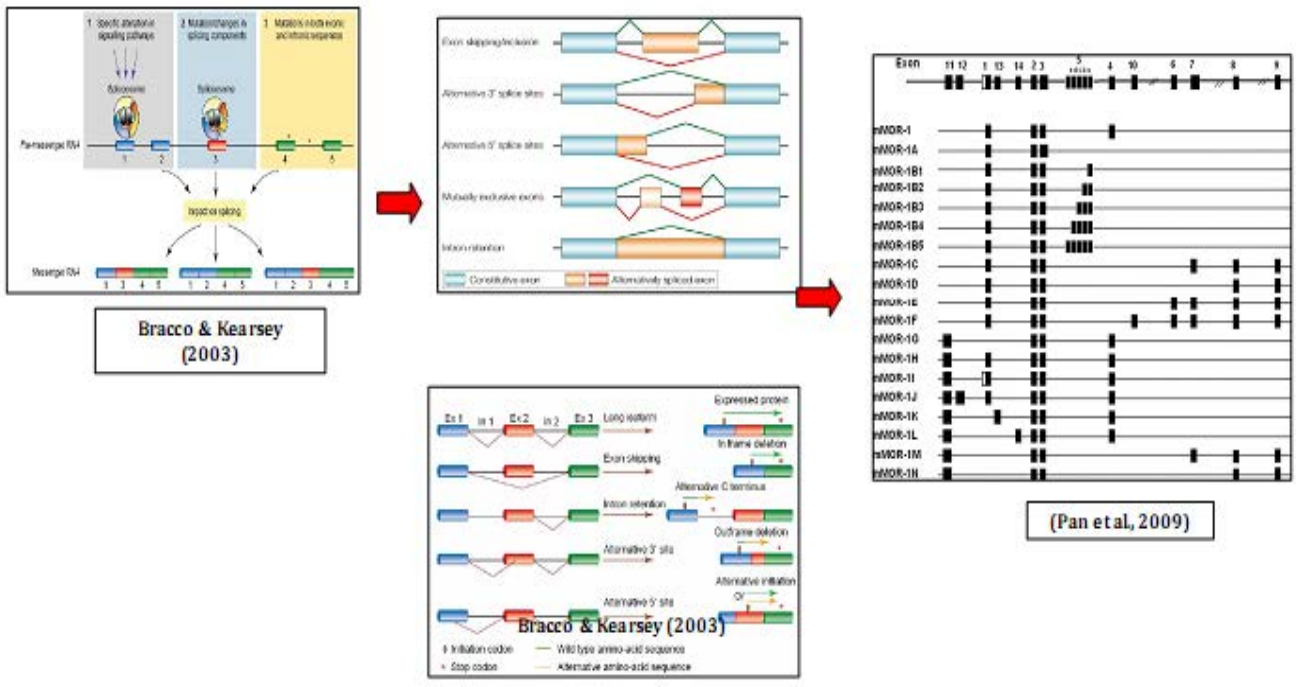


Figure 3: Schematic Representation of the Role of Alternative Splicing in Mu-opioid Receptor (MOR-1) Physiology

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Virtishir™ Homogenizer



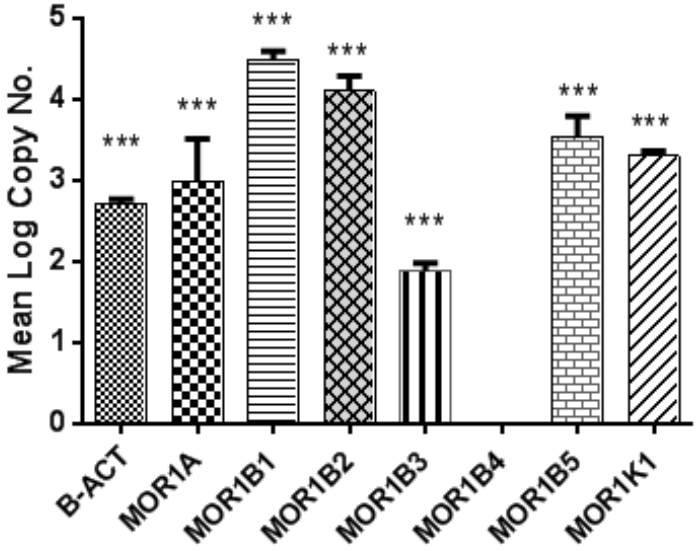
Nanodrop™



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Figure 4: Some Instruments of Gene Expression Analysis.

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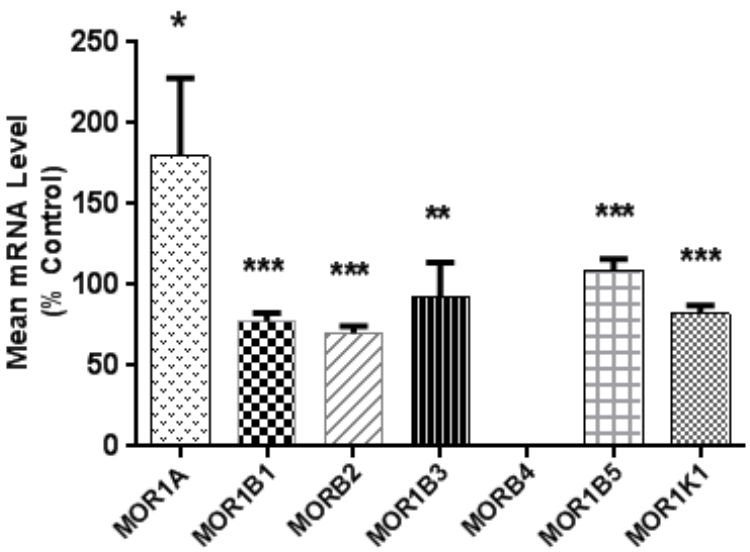
		Mean Log Copy No.		SEM	n
Control	BACT	2.719	±	0.05	3
	MOR1A	2.993	±	0.52	3
	MOR1B1	4.491	±	0.11	3
	MORB2	4.103	±	0.19	3
	MOR1B3	1.883	±	0.10	3
	MORB4	ND			3
	MOR1B5	3.531	±	0.27	3
	MOR1K1	3.310	±	0.05	3

*** P<.0001

[#] Represents number of independent samples. Each sample was run in triplicate.
SEM – standard error of the mean; n – number of samples

Figure 5: Relative Expression of MOR1 Splice Variant mRNA expression in Untreated, Retinoic Acid (RA)-differentiated (Control) Human Neuroblastoma (SH-SY5Y) Cells

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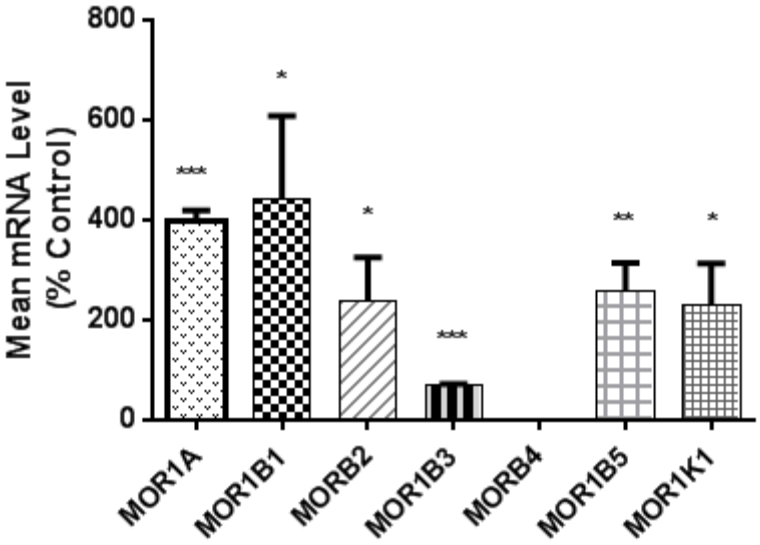


	Gene	Mean (% Control)		SEM	n#
Morphine	MOR1A	179.7	±	48.1	2##
	MOR1B1	77.3	±	5.3	3
	MORB2	70.1	±	4.4	3
	MOR1B3	92.9	±	20.9	3
	MORB4	ND			
	MOR1B5	109.3	±	6.7	3
	MOR1K1	82.2	±	4.9	3

* p<.05; ** p<.005; *** p<.0001
# Represents number of independent samples Each sample was run in triplicate.
Effect not detected in remaining sample for MOR1A.
SEM – standard error of the mean; n – number of samples

Figure 6: Effect of Morphine on Mu-opioid Receptor (MOR-1) Alternatively-spliced Variants Expression in Retinoic Acid (RA)-differentiated Human Neuroblastoma (SH-SY5Y) Cells

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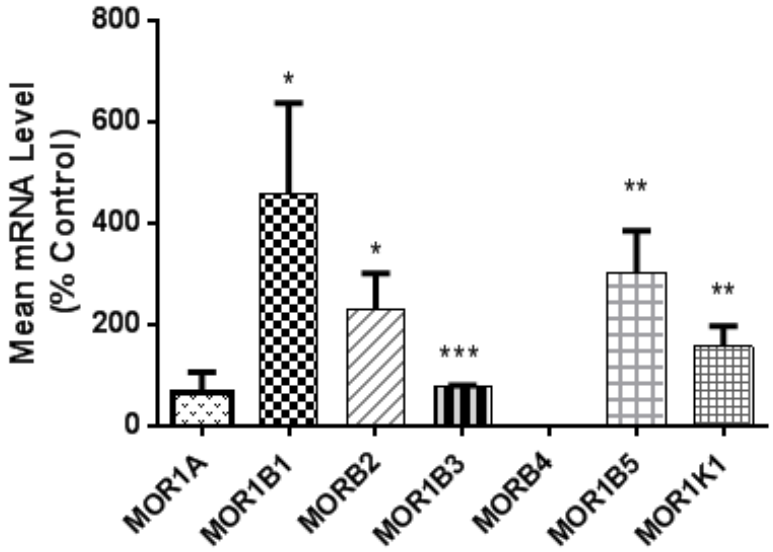


	Gene	Mean (% Control)		SEM	n#
Alma Fig	MOR1A	396.1	±	22.8	3
	MOR1B1	440.1	±	168.0	3
	MORB2	239.1	±	85.8	3
	MOR1B3	69.9	±	2.2	1##
	MORB4	ND			
	MOR1B5	259.1	±	55.2	3
	MOR1K1	230.2	±	82.5	3

* p<.05; ** p<.005; *** p<.0001, p=.0009
# Represents number of independent samples. Each sample was run in triplicate.
Effect not detected in remaining two samples.
SEM – standard error of the mean; n – number of samples

Figure 7: Effect of Alma Fig (*Ficus carica*) Leaf Extract on Mu-opioid Receptor (MOR-1) Alternatively-spliced Variants Expression in Retinoic Acid (RA)-differentiated Human Neuroblastoma (SH-SY5Y) Cells

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	Gene	Mean (% Control)		SEM	n#
Morphine/ Fig	MOR1A	65.4	±	40.7	2##
	MOR1B1	459.6	±	177.4	3
	MORB2	228.1	±	72.8	3
	MOR1B3	77.0	±	3.0	2##
	MORB4	ND			
	MOR1B5	301.8	±	83.7	3
	MOR1K1	156.6	±	40.5	3

p<.05; ** p<.005, P=.0069; *** p<.0001
Represents number of independent samples. Each sample was run in triplicate.
Effect not detected in remaining sample(s).
SEM – standard error of the mean; n – number of samples

Figure 8: Combined Effect of Morphine Plus "Alma" Fig Leaf Extract on the Expression of Mu-opioid Receptor (MOR- 1) Alternatively-spliced Variants in Human Neuroblastoma (SH-SY5Y) Cells

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Tables

Table 1: Characteristics of Selected *Ficus carica* L. Cultivars

Cultivar (Variety)	Description of Leaf[1]	Description of Fruit[1,2]	Origin[3]	Leaf Extract Pigment (pH)
Alma	The leaf has a decurrent base and is unlobed to tri-lobed.	Golden brown skin; amber-tan flesh; small eye; shape is pyriform with a neck.	USA (Released by Texas Agricultural Experiment Station, Texas A&M Univ., 1974); cross between female Allison and male Hamma Caprifig.	Green-yellow (6.01, 6.03)
Black Jack	Similar to San Piero	Large to very large, purple-brown fig; oblate in shape.	USA	Bright green (pH 5.52, 5.53)
Brown Turkey (*San Piero; California Brown Turkey)	The leaf has a subcordate base with 3 lobes and a crenate margin. (The leaf has a calcarate base with lyrate lobes and a crenate margin.) Antiooxidants[5]: polyphenols, flavonoids, anthocyanins	Medium to large sized, bell shaped, purplish brown fig; small eye; pinkish amber flesh; turbinate to oblique mostly without necks. (Very large; red flesh; breba fruit are oblique-pyriform, sometimes elongated; main crop fruit are obolate to oblique-pyriform with variable necks; typically flattened at the eye end; eye is large and open)	Spain (Clone introduced in 1769)	Dark green (pH 7.02, 7.03)
Celeste (Malta)	The leaf has a subcordate base, 3 to 5 lobes and crenate margins.	Small, brown to purple in color[2]; tightly closed eye. (Light brown to violet-brown; flesh is reddish-amber in color; pyriform with tapering neck.)	France	Color N (pH 5.96, 5.97)R
Giant Celeste (Tiger)	Has large palmate leaves with 5 to 7 lobes. The middle lobe is spatulate and the margins are slightly toothed.	Large, brown fig with short neck; partially closed eye; yellow to gold pulp;	USA (LSU)	Dark green, brown (pH 6.51, 6.52)
Green Ishia (Verte)	Leaf has broadly subcordate to truncate base and 3 lobes.	Medium-sized, green to greenish-yellow, thin-skinned fig; red flesh resembling strawberry; shape is oblate to spherical.	Spain	Dark green (pH 5.69, 5.70)
Hollier	The leaf has a cordate base and 5 latate lobes.	Greenish-yellow skin; amber pulp tinged strawberry; Oblate-spheroid shape	USA (LSU)	Dark green (pH 6.30,6.31)
Mission (Franciscana)	The leaf has a cacarate base and 5 latate lobes. Phytosterols[4]: campesterol, stigmasterol, sitosterol, fucosterol. Antiooxidants[5]: polyphenols (higher), flavonoids (higher), anthocyanins (highest).	Huge, pear-shaped, purple-black fig; pink flesh; breba fruit are pyriform with prominent, thick necks; main crop is smaller with more variable necks	Spain	Dark green (pH 5.13, 5.14)

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Nero (Barnisotte)	Leaf has a cordate base and 5 lobes with the middle one being spatulate and the others latate.	Large, reddish black fig; eye is medium-sized and open; shape is turbinate-pyriform, sometimes oblique with a broad apex.	Italy	Dark green (pH 5.78, 5.79)

[1] Source: www.durionursery.biz/figs.htm
[2] Source: Aggie Horticulture, online at http://aggie-horticulture.tamu.edu/extension/homefruit/fig/fig.html
[3] Source: Aradhya et al. (2010).
[4] Jeong & Lachance (2001).
[5] Bekoe et al. (2011)
NR = not recorded

Table 2: Oligonucleotide Primers Used for qRT-PCR Analysis

Primer	Type	Sequence (5' to 3')	Bases	G/C Count
hMOR-1 1F	Forward (Sense)	ATGCCAGTGCTCATCATTAC	20	9
hMOR-1 1R	Reverse (Antisense)	GATCCTTCGAAGATTCCTGTCCT	23	11
hMOR-1A 1F	Forward (Sense)	CAGGTACGCAGTCTCTAGAATTAGG	25	12
hMOR-1A 1R	Reverse (Antisense)	TTCCCTCCATTCTCATCCTC	20	10
hMOR-1B1 1F	Forward (Sense)	TCAAAAGTCATCTTTACTCAACTGTG	26	9
hMOR-1B1 1R	Reverse (Antisense)	GCTTCCAATCTTATATTCTTTCACG	25	9
hMOR-1B2 1F	Forward (Sense)	AAAGAAGACAGAAATCTGACTGGTAA	26	9
hMOR-1B2 1R	Reverse (Antisense)	GCAAGCCGGATCACTAGG	18	11
hMOR-1B3 1F	Forward (Sense)	TTTGTTGCTGACCAACTTGC	20	9
hMOR-1B3 1R	Reverse (Antisense)	GGTCGTTTTTCTGTGTTGAGG	21	10
hMOR-1B5 1F	Forward (Sense)	GGAATTGAACCTGGACTGTCA	21	10
hMOR-1B5 1R	Reverse (Antisense)	AAGCCTTCGCAAACTCAAAA	20	8
hMOR–1K1 1F	Forward (Sense)	CTGGGTAGGAAAGTGGCAAA	20	10
hMOR-1K1 1R	Reverse (Antisense)	TGACCTTGGTGCTCAAGAAGT	21	10

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Table 3: General Characteristics of Receptors

Type	Characteristics[1]
Nature[2]	Mostly protein – lipoprotein, glycoprotein
Location	Mostly on the cell surface; some intracellular[2]
Molecular Mass	≈45 to 200 kilodaltons; may consist of subunits
Dissociation Constant (K_d)	Binding capacity – 1 to 100 nM
Reversibility	Binding to the ligand-binding domain is reversible (non-covalently bound) and stereospecific. (Neurotransmitters, hormones, and most drugs act in a reversible manner. Hence, binding should also be reversible.)[3]
Specificity of binding	Affinity not absolute; drug may bind to several receptor types (a continuum)
Saturability	Affirmative due to finite number of receptors
Drug Specificity	Specific binding to receptor triggers signal transduction to intracellular site
Ligand	>1 Drug molecule may be required to bind in order to generate a signal. Binding of a ligand to a receptor should be dissociable and recoverable in its natural (non-metabolized) form[4].
Signal Intensity	Magnitude of signal depends on number of receptors occupied or on receptor occupancy rate; signal is amplified by intracellular mechanisms
Drug-Receptor Interaction	By acting on a receptor, drugs can enhance, diminish, or block generation or transmission of signal
Drug-Cell or Drug-Tissue Interaction	Drugs are receptor modulators and do not confer new properties on cells or tissues.
Essential Properties	Receptors must have properties of recognition and transduction.
Effect on Receptor Numbers	Receptors can be up-regulated or down-regulated.
Restoration of Function Upon Reconstitution[2]	An authentic receptor should be recoverable in its natural (non-metabolized) form. If the gene for such receptor is isolated and expressed, it should be exactly similar to the cloned receptor of the natural receptor.

[1] Adopted from Hollinger (1997)

[2.] Receptors can contain secondary modifications of carbohydrate and be selectively embedded into the lipid membrane bilayer (Norman & Litwack, 1997).

[3] Regardless of where they have been isolated from, studies show that neurotransmitter and peptide hormone receptors are localized on the cell surface. Steroid and thyroid hormones are located intracellulary with the nucleus and cytoplasm, respectively (Cooper et al., 2003).

[4.] Adopted from Cooper et al., 2003

[5.] All receptors have an effector domain that "recognizes" the presence of the hormone bound to the ligand domain and that then initiates the generation of the biological response(s) (Norman & Litwack, 1997).

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Stability Indicating RP-HPLC Method Development and Validation for Simultaneous Estimation of Rivaroxaban and Clopidogrel Bisulphate in Pharmaceutical Dosage Form

By N. I. Majan, N. I. Patel & Aejaz Ahmed
Ali-Allana College of Pharmacy

Abstract- A simple, specific, accurate and stability-indicating reversed phase high performance liquid chromatographic method was developed for the simultaneous determination of Rivaroxaban and Clopidogrel, using a C18 (25cm x 0.46 cm) Hypersil BDS column and a mobile phase composed of buffer (pH 4.5): methanol (70:30). The detection was carried out at wavelength 214 nm. The retention times of Rivaroxaban and Clopidogrel were found to be 3.300 min and 4.740min, respectively. Linearity was established for Rivaroxaban and Clopidogrel in the range of 2-6µg/ml and 7.5-22.5µg/ml, respectively. The percentage recoveries of Rivaroxaban and Clopidogrel were found to be 100.09% and 99.79%, respectively. Both the drugs were subjected to acid, alkali, oxidation, thermal and photolytic UV degradation. The degradation study shows that both drugs are susceptible in all parameter. Clopidogrel is more susceptible for photo and thermal degradation.

Keywords: clopidogrel bisulphate, rivaroxaban, rp-hplc, stability indicating method.

GJMR-B Classification: NLMC Code: QV 704



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Keywords: clopidogrel bisulphate, rivaroxaban, rp-hplc, stability indicating method.

I. Introduction

Rivaroxaban ((S)-5-chloro-N-{[2-oxo-3-[4-(3-oxo-morpholin-4-yl) phenyl] oxazolidin-5-yl] methyl} thiophene-2-carboxamide) (fig. 1) is an anticoagulant and the first orally active direct factor Xa inhibitor [1]. Clopidogrel Bisulphate methyl (2S)-2-(2-chlorophenyl)-2-{4H, 5H, 6H, 7H-thieno [3, 2-c] pyridin-5-yl} acetate (fig. 2)is an antiplatelet agent structurally and pharmacologically similar to ticlopidine. It is used to inhibit blood clots in a variety of conditions such as peripheral vascular disease[2].

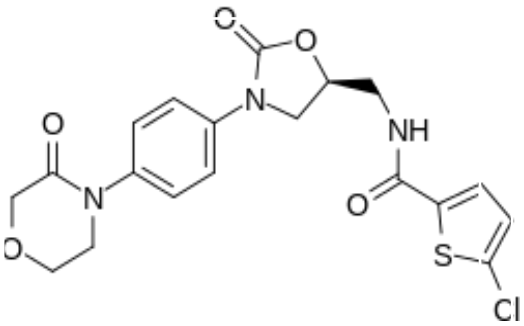


Figure 1: Structure of Rivaroxaban

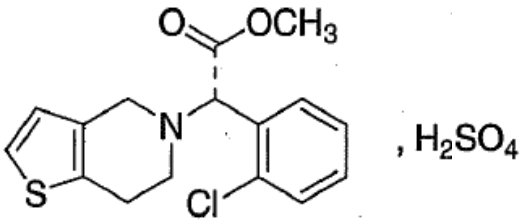


Figure 2: Structure of Clopidogrel Bisulphate

Fixed dose combination (FDC) for the probing drugs is not available commercially, yet this FDC has not been listed in any of the common pharmacopoeia. When go through the literature lead to the occurrence of various singular methods for this drug like, for Rivaroxaban RP-HPLC[3-7], RP-HPLC and UPLC (Forced Degradation study)[8], RP-UPLC (Stability indicating Method)[9], RP-HPLC (Stability indicating Method)[10], RP-HPLC (Stability indicating Dissolution Method)[11], RP-HPLC and TLC[12], HPTLC[13], UV Spectrophotometry[14] and for Clopidogrel Bisulphate Chiral Chromatography[15-16], RP-HPLC[17-20], Stability Indicating RP-

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HPLC[21-22]. In combination of this drug only RP-HPLC method were available in mobile phase and also in plasma and urine [23-24-25]. However no stability indicating RP-HPLC method was set up for this combination.

We are involved newly to conduct investigation relating to stability indicating RP-HPLC method development of Rivaroxaban and Clopidogrel for fill this information gap. It was tried to develop and validate RP-HPLC method with stability indicating properties for this combination (Rivaroxaban and Clopidogrel Bisulphate). We expect the inclusion of this knowledge in the current literature will be benefit for the pharmaceutical industries for support the quality of their products holding these active ingredients and also the execution agencies in broad to evaluate the quality of the marketed preparations.

II. Experimental

Chemical and reagent: Pure Clopidogrel (CLP) and Rivaroxaban (RIV) were obtained as a gift sample from Remus Remedies. As Sample Clopidogrel 75mg and Rivaroxaban 20mg Synthetic Mixture is used. HPLC grade Methanol, Potassium dihydrogen, Ammonium Acetate, HPLC Grade High purity deionized water were obtained from Merck specialties Pvt Ltd., Mumbai.

Instrumentation and materials: The liquidchromatographic system was of Thermo separation Product TSP UV 2000, which consisted a gradient pump, variable wavelength, programmable UV/Vis detector, a manual injection facility with 20 µl fixed loop. The chromatographic analysis was performed using spinchrom software on a C18 Hypersil BDS column (25cm x 0.46 cm with 5µm particle size). In addition, an electronic balance (CP-124S Sartorius, Germany), a pH meter (Electroquip's Digital pH meter)were used in this study.

Chromatographic conditions: The elution of CLP and RIV was obtained by running HPLC in isocratic mode using Phosphate Buffer (pH 4.5): Methanol (70:30).Flow rate was maintained at 1.0 ml/min with run time of 6 min. The retention time for RIV was obtained 3.300 minand CLP was obtained 4.740min. Detection was performed at 214 nm. Mobile phase was previously filtered through Whatman filter paper no 41.

a) Preparation of standard solutions

CLP standard stock solution (150µg/ml): A 15 mg of CLP was weighed and transferred to a 100 ml volumetric flask. Volume was made up to the mark with mobile phase.

RIVstandard stock solution(40µg/ml): A 40 mg of RIV was weighed and transferred to a 100 ml volumetric flask. Volume was made up to the mark with mobile phase, taken 10ml from this solution and transferred to 100ml volumetric flask and volume was made up with methanol.

Preparation of standard solution of binary mixtures of CLP (15 µg/ml) and RIV(4 µg/ml): Take 1 ml from the CLP stock solution and 1ml from RIV stock solution and transferred to 10 ml volumetric flask and volume made up to the mark by mobile phase which was used in particular trials.

b) Preparation of formulation solution

Sample Stock Solution (CLP 150 µg/ml, RIV 40 µg/ml): Take Tablet powder equivalent to 15 mg of CLP and 4 mg of RIV was transferred to a 100 ml volumetric flask, Add 60 ml Mobile phase and Shake for 15 min and make up volume with Mobile phase. The solution was filtered through Whatman filter paper no. 42.

Working Sample Preparation (CLP 15 µg/ml, and RIV 4 µg/ml): Take 1 ml from standard stock solution and transferred to 10ml volumetric flask and made up volume up to the mark with the mobile phase.

c) Forced degradation study [26]

Acid degradation: Acid decomposition studies were performed by transferring 1 ml of stock solution in to 10 ml of volumetric flask. 2ml of 0.1 N Hydrochloride solutions was added and mixed well and put for 5 hrs. at room temperature. Then the volume was adjusted with diluent to get 15µg/ml for CLP and 4µg/ml for RIV.

Base degradation: Basic decomposition studies were performed by transferring 1ml of stock solution in to 10ml of volumetric flask. 2 ml of 0.1 N NaOH solutions was added and mixed well and put for 3 hrs at room temperature. Then the volume was adjusted with diluent to get 15µg/ml for CLP and 4µg/ml for RIV.

Oxidative degradation: Oxidative decomposition studies were performed by transferring 1ml of stock solution in to 10 ml of volumetric flask. 2 ml of 3% H_2O_2 solutions was added and mixed well and put for 6 hrs at room temperature. Then the volume was adjusted with diluent to get 15µg/ml for CLP and 4µg/ml for RIV.

Photo degradation: Photo degradation studies were performed by transferring 1ml of stock solution in to 10 ml of volumetric flask. The volumetric flask was kept in UV Chamber for 12 hrs. Then the volume was adjusted with diluent to get 15µg/ml for CLP and 4µg/ml for RIV.

Thermal degradation: Thermal degradation studies were performed by transferring 1ml of stock solution in to 10 ml of volumetric flask. The volumetric flask was stored in oven at 80°C for 5 hrs. Then the volume was adjusted with diluent to get 15µg/ml for CLP and 4µg/ml for RIV.

Method validation: After method development, the method was validated in compliance with ICH guidelines. The method was validated for Accuracy, Precision, Reproducibility, Specificity, Limit of Detection, Limit of Quantitation, Linearity and Range, Ruggedness and Robustness.

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III. Result and Discussion

a) Optimization of mobile phase

Trial contains various mobile phase which are considered of Methanol, Water and buffer. Methanol in different proportions and different volumes at different flow rate were tried. On the basis of various trials the mixture of Buffer (pH 4.5): Methanol (70:30), at 1.0 mL/min flow rate, proved to be better than the other mixture in terms of peak shape, theoretical plate and asymmetry.

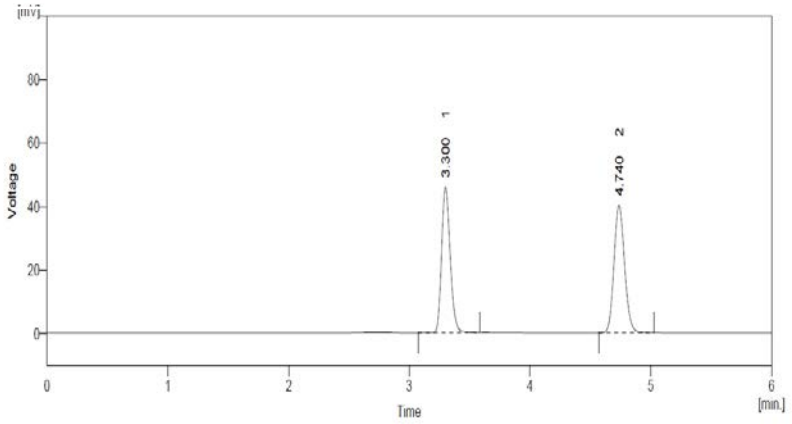


Figure 3: HPLC Chromatogram of RIV and CLP Buffer (pH 4.5): Methanol (70:30)

b) System suitability parameter

System suitability is an integral part of chromatographic method. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole. System suitability testing provides assurance that the method will provide accurate and precise data for its intended use. Observed values for system suitability is show in Table 1.

Table 1: Result of system suitability parameters

Parameters	RIV	CLP
Retention Time	3.300	4.740
Theoretical Plates	9427	13320
Asymmetry	1.316	1.160
Resolution	9.593	

c) Validation of RP – HPLC method

i. *Specificity*

The Chromatograms of Clopidogrel and Rivaroxaban standards and Clopidogrel and Rivaroxaban sample show no interference with the Chromatogram of Clopidogrel and Rivaroxaban Blank, so the Developed method is Specific.

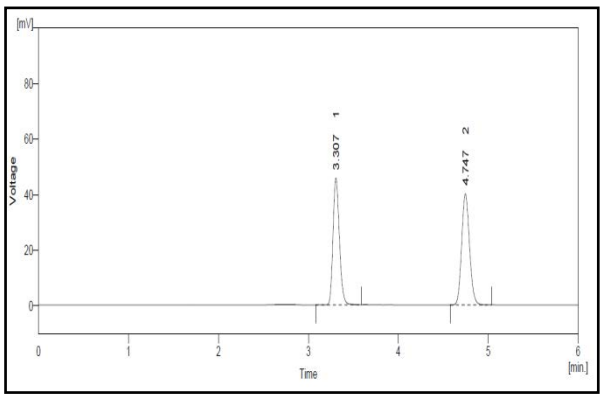


Figure 4: Chromatogram of RIV and CLP standard

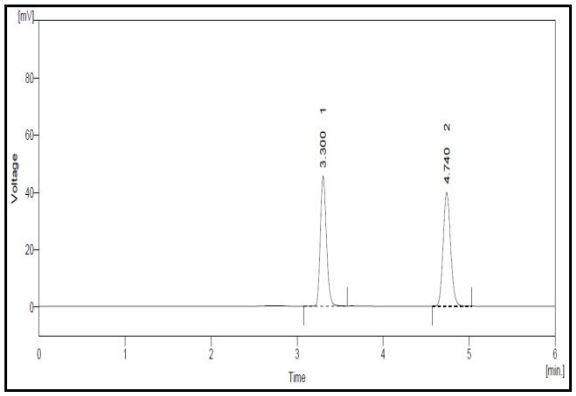


Figure 5: Chromatogram of RIV and CLP sample

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d) Linearity

The linearity for RIV and CLP were assessed by analysis of combined standard solution in range of 2-6 µg/ml and 7.5-22.5 µg/ml respectively, Correlation co-efficient for calibration curve RIV and CLP was found tobe0.998 and 0.999 respectively The regression line equation for RIV and CLP are as following:

For RIVy = 55.905x + 0.2138 and for CLP y = 16.145x - 0.4796

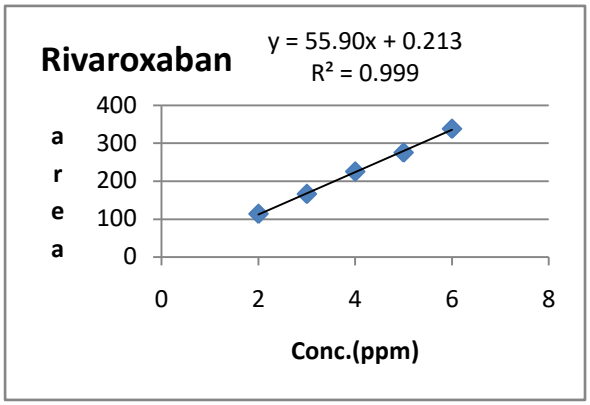


Figure 6: Calibration Curve of RIV

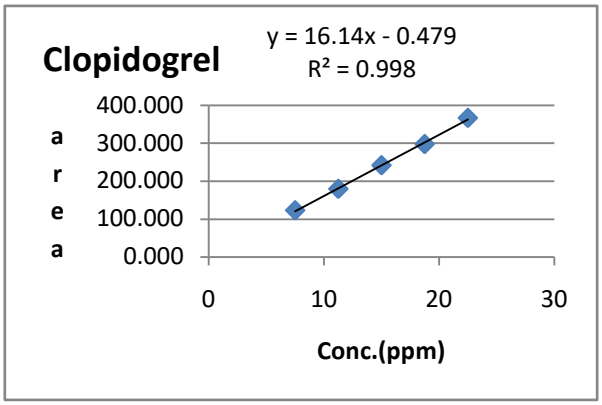


Figure 7: Calibration Curve of CLP

e) Precision

The precision of the method was demonstrated by repeatability study, inter-day precision and intra-day precision. In the repeatability study, six replicates of the same concentration of working standard solutions were prepared and injected and chromatograms were recorded. The results obtained were shown in Table 2. In inter-day precision and intra-day precision, three replicates of three different concentration of working standard solution were prepared and injected and chromatograms were recorded. The results obtained were shown in Table 3.

Table 2: Results of repeatability study

Drugs	Mean Area (n=6)	S.D	%R.S.D
RIV	224.773	1.058	0.471
CLP	242.442	1.451	0.599

n- Number of estimations

Table 3: Results of intra-day and inter-day precision

Parameter	Drug	Amount Taken (µg mL-1)	Mean Area Found (n=3)	S.D	%R.S.D
Intraday precision	RIV	2	113.066	0.556	0.451
		4	225.837	1.696	0.751
		6	335.157	1.21	0.368
	CLP	7.5	122.28	0.754	0.616
		15	243.516	1.319	0.541
		22.5	362.858	1.696	0.467
Interday precision	RIV	2	112.632	0.972	0.863
		4	224.325	1.237	0.552
		6	333.162	3.512	1.054
	CLP	7.5	122.769	0.388	0.316
		15	241.954	1.786	0.738
		22.5	360.46	4.525	1.255

n- Number of estimations

Accuracy

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Accuracy of the method was confirmed by recovery study from formulation at three level of standard addition.2 µg/ml drug solution for RIV and 7.5µg/ml drug solution for CLP was taken in three different flask label A, B and C. Spiked 80%, 100%, 120% of standard solution in it and diluted up to 10ml. The area of each solution peak was measured at 214 nm. The amount of RIV and CLP was calculated at each level and % recoveries were computed. The results are shown in table 4. Percentage recovery for Rivaroxaban was 0.718%-1.357 %, while for Clopidogrel it was found to be in range of 0.649 %-1.110 %

Table 4: Results of recovery study

Drug	Conc. Level(%)	Sample amount added(µg/ml) (n=3)	Standard amount added(µg/ml) (n=3)	Mean of amount recovered (µg/ml) (n=3)	% Mean recovery	S.D	% R.S.D
RIV	80	2	1.6	1.601	100.081	1.358	1.357
	100	2	2	1.992	99.594	0.716	0.718
	120	2	2.4	2.415	100.623	0.734	0.729
CLP	80	7.5	6	6.001	100.028	0.649	0.649
	100	7.5	7.5	7.474	99.656	0.948	0.951
	120	7.5	9	8.975	99.728	1.107	1.11

n- Number of estimations

LOD and LOQ

Calibration curve was repeated for five times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were calculated as follows:

LOD = 3.3 * SD/slope of calibration curve

LOQ = 10 * SD/slope of calibration curve

Where, SD = Standard deviation of intercepts

Table 5: Results of LOD and LOQ

LOD		LOQ	
RIV	CLP	RIV	CLP
LOD=3.3x(SD/Slope) = 3.3 x(3.302/55.905) = 0.195 µg/ml	LOD=3.3x(SD/Slope) = 3.3 x(3.849/16.145) = 0.787 µg/ml	LOQ=10x(SD/Slope) = 10 x(3.302/55.905) = 0.591 µg/ml	LOQ=10x(SD/Slope) = 10 x(3.849/16.145) = 2.384 µg/ml

f) Robustness

Following parameters were changed one by one and their effect was observed on system suitability for standard preparation. (a)Flow rate of mobile phase was changed (± 0.2 ml/min) 0.8 ml/min and 1.2 ml/min. (b) Ratio of Mobile phase was changed (±2) Buffer: Methanol (72:28) and Buffer: Methanol (68:32). (c)pH of Buffer was changed (±0.2), pH 4.3 and pH 4.7.The effect of changes was found to be within the acceptance criteria as shown in table 6. The % RSD should be less than 2%.

Table 6: Results of robustness study

Parameter	Variation	RIV			CLP		
		Mean area (n=3)	S.D	%R.S.D	Mean area (n=3)	S.D	%R.S.D
Mobile phase	72:28	229.51	1.036	0.451	246.529	3.309	1.342
	68:32	217.964	0.579	0.266	234.914	0.236	0.101
Flow rate	0.8 ml/min	233.192	3.135	1.344	252.901	1.553	0.614
	1.2ml/min	213.584	0.697	0.326	230.47	0.907	0.394
pH	4.3	233.898	3.193	1.365	240.119	0.636	0.265
	4.7	222.218	1.783	0.802	252.359	1.409	0.558

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g) Assay

Triturate 20 tablets, take tablet powder equivalent to 15 mg of CLP and 4 mg of RIV was transferred to a 100ml volumetric flask, Add 60 ml Mobile phase and Shake for 15 min and make up volume with Mobile phase. Take 1 ml from this stock solution and transferred to 10 ml volumetric flask and made up volume up to the mark with the mobile phase. Inject above Solution 20 µl for assay analysis. The solution was filtered through What man filter paper no. 42. The results are given in Table 7.

Table 7: Results of assay study

Tablet		
Label claim	CLP(75mg)	RIV(4mg)
Assay (% of label claim*) Mean ± S. D.	98.895±0.267	98.651±0.363

h) Stability indicating method

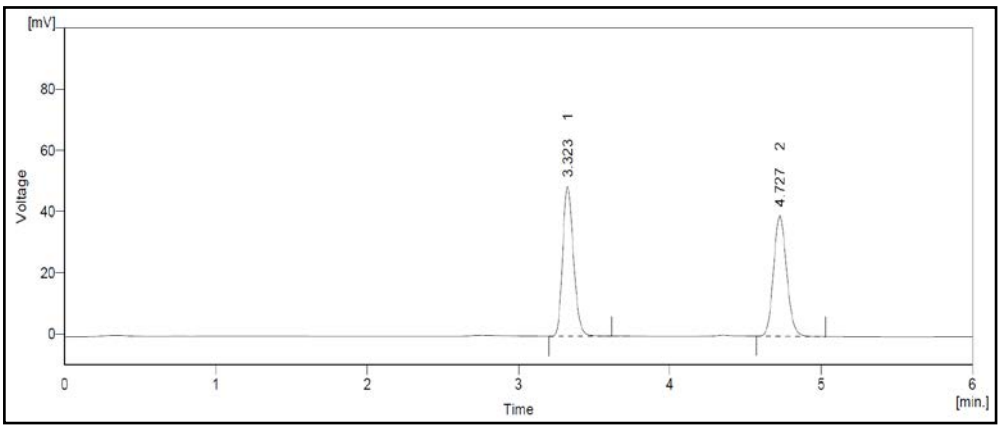


Figure 8: RIV and CLP Standard for stability

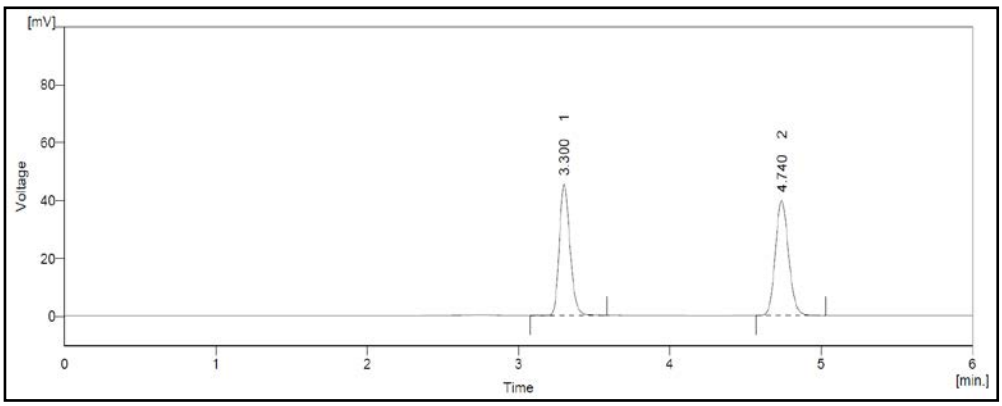


Figure 9: RIV and CLP sample

Table 8: Calculation of CLP and RIV standard for stability

Drugs	Area
Rivaroxaban	242.859
Clopidogrel	237.109

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i) Acid degradation

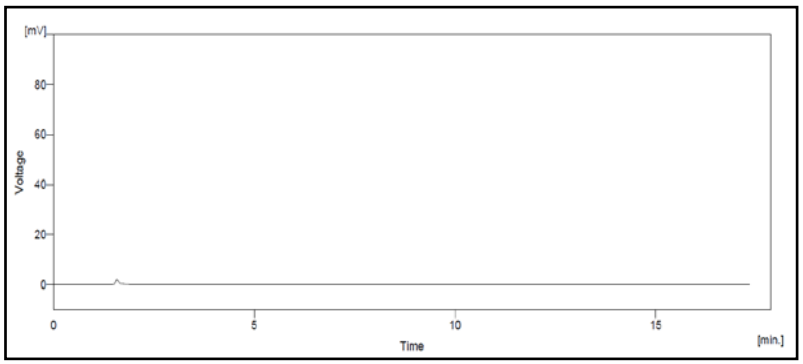


Figure 10: Acid degradation blank

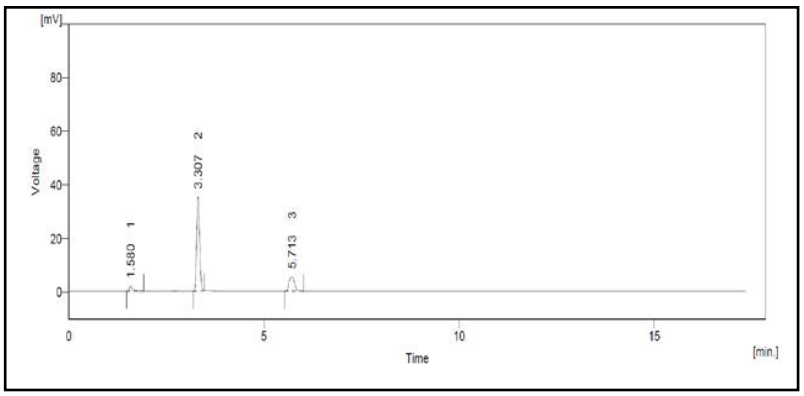


Figure 11: RIV acid degradation standard

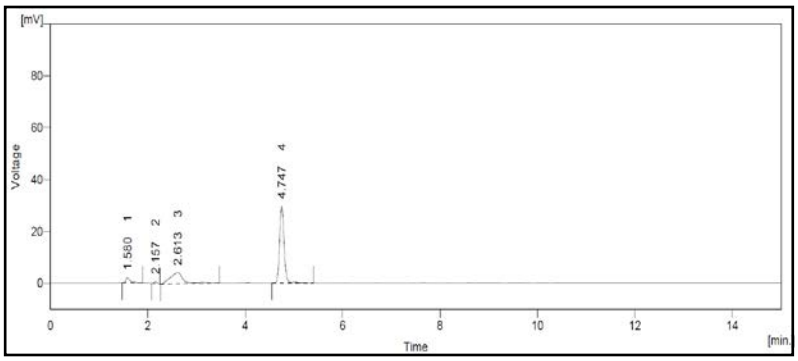


Figure 12: CLP acid degradation standard

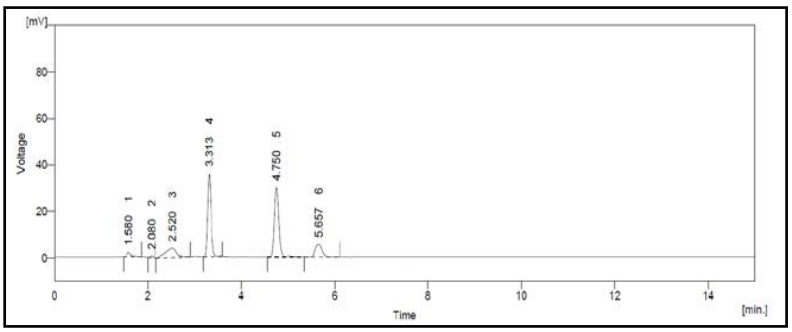


Figure 13: RIV and CLP acid degradation sample

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j) Base degradation

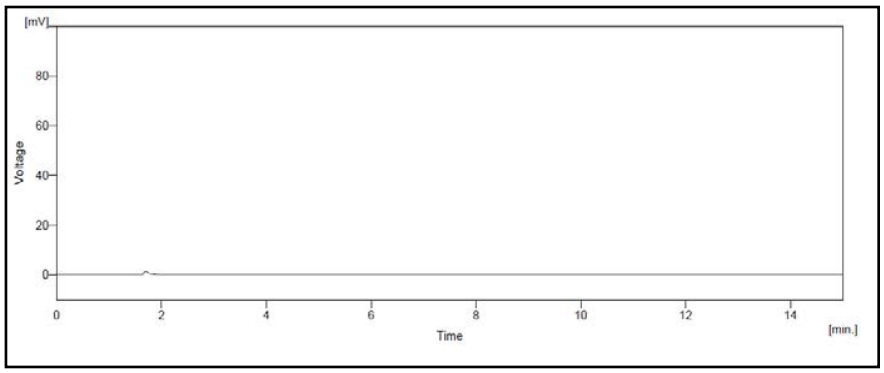


Figure 14: Base degradation blank

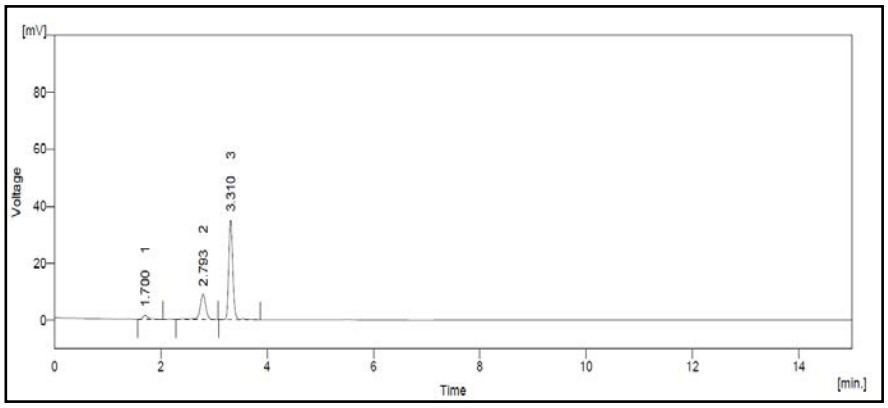


Figure 15: RIV base degradation

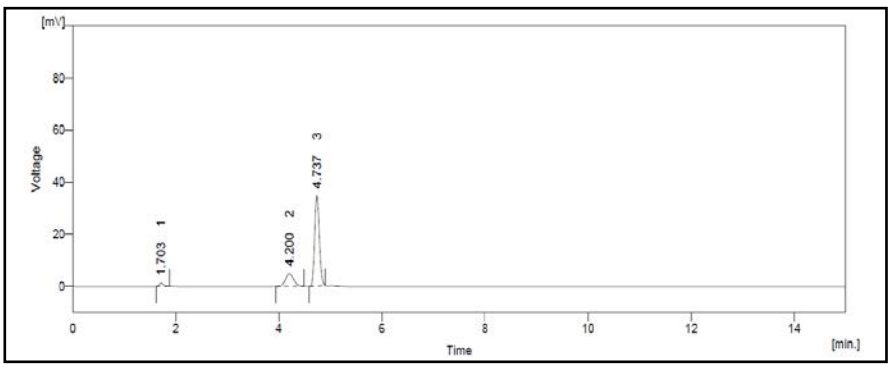


Figure 16: CLP base degradation

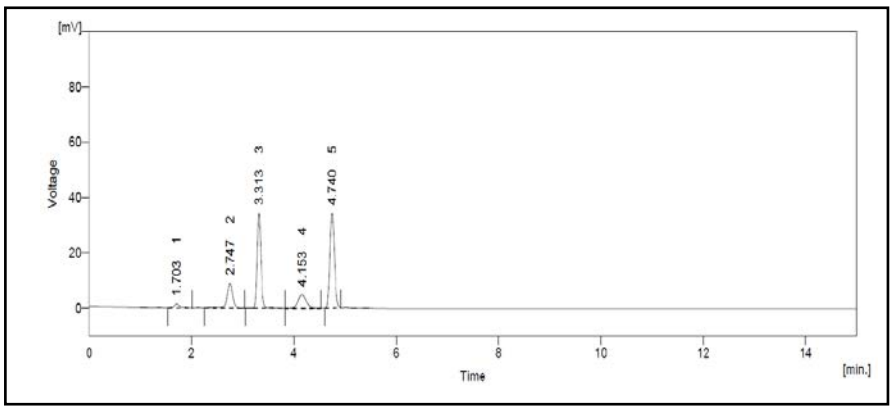


Figure 17: RIV and CLP base degradation sample

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k) Oxidation Degradation

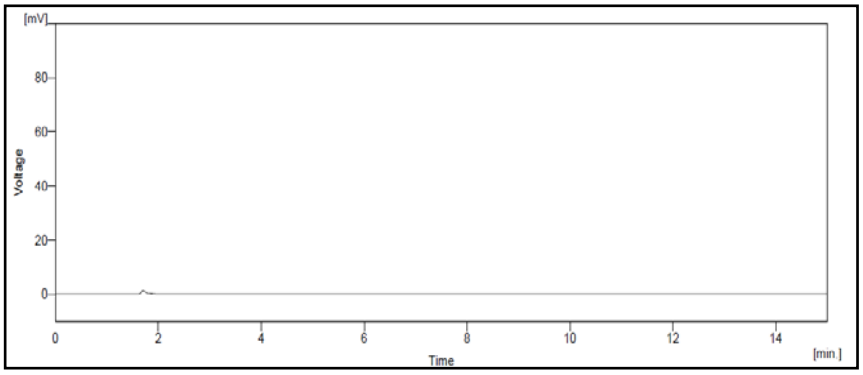


Figure 18: Oxidation degradation blank

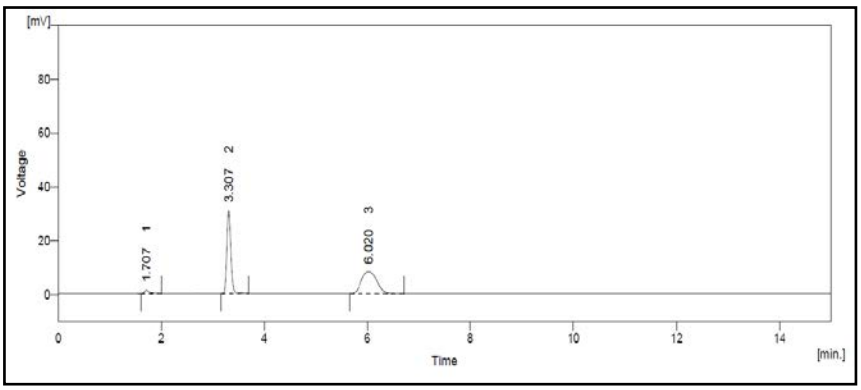


Figure 19: RIV oxidation degradation

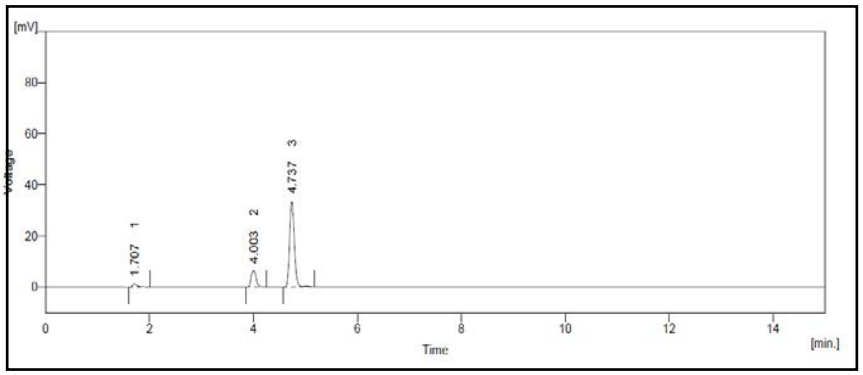


Figure 20: CLP oxidation degradation

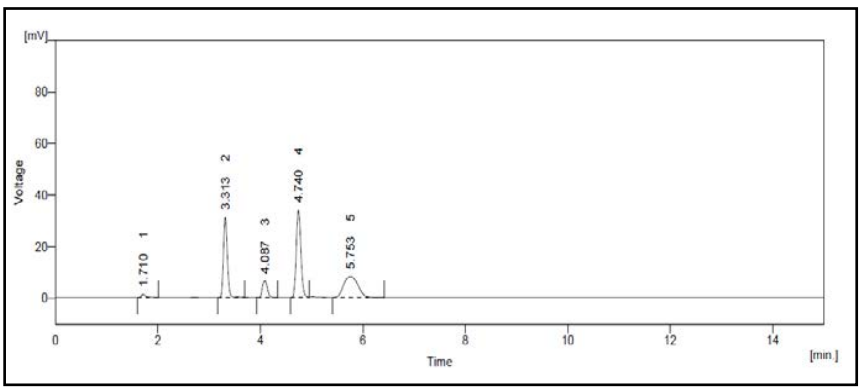


Figure 21: RIV and CLP oxidation degradation sample

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I) Photo degradation

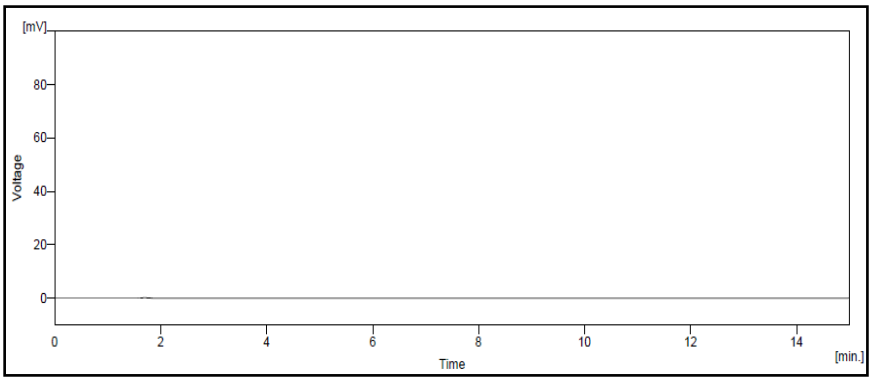


Figure 22: Photo degradation blank

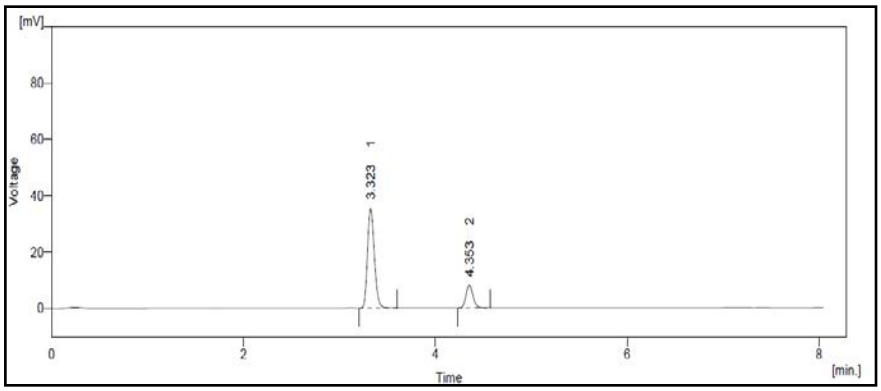


Figure 23: RIV photo degradation

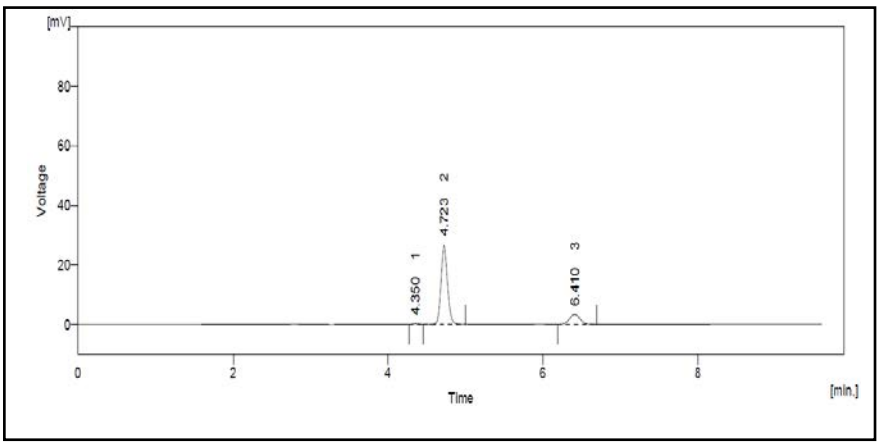


Figure 24: CLP photo degradation

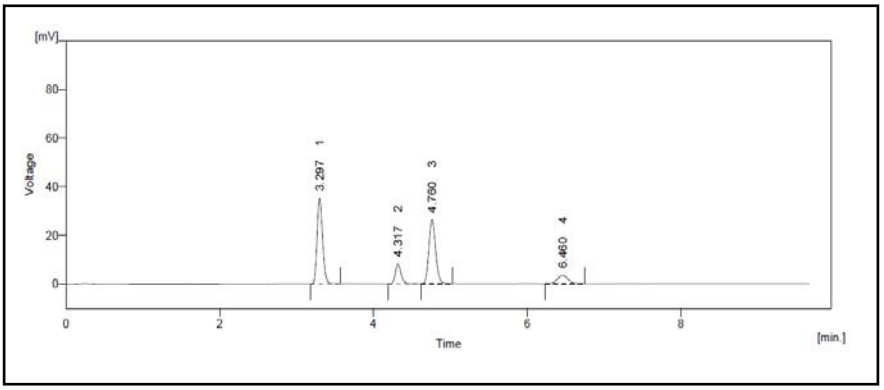


Figure 25: RIV and CLP photo degradation sample

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m) Thermal degradation

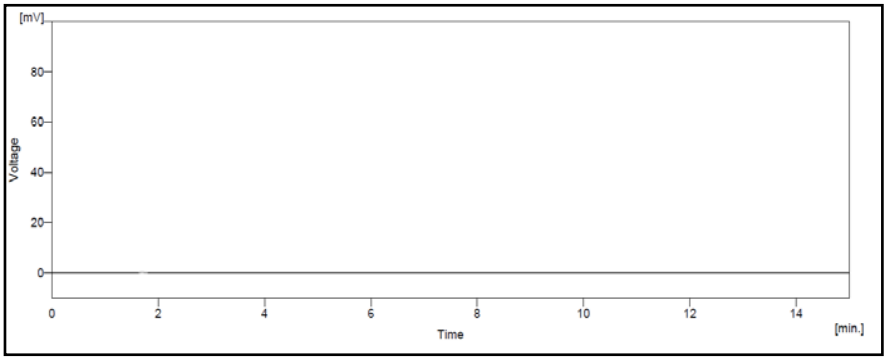


Figure 26: Thermal degradation blank

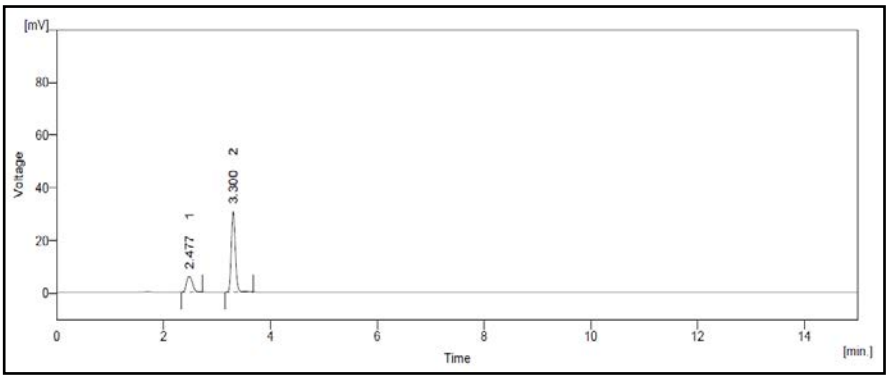


Figure 27: RIV thermal degradation

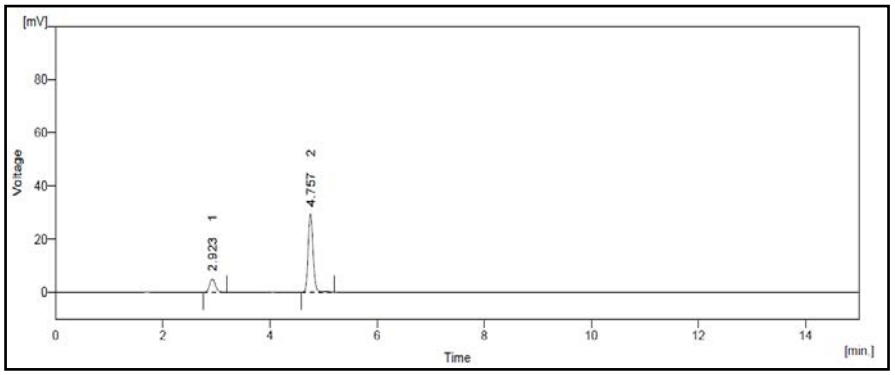


Figure 28: CLP thermal degradation

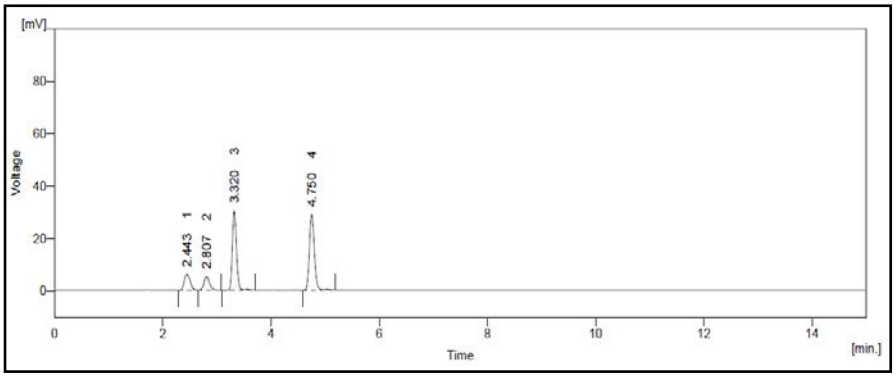


Figure 29: RIV and CLP thermal degradation sample

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Table 9: Calculation of CLP and RIV% degradation study

Parameter	CLP				RIV			
	Standard		Sample		Standard		Sample	
	Area	% Degradation	Area	% Degradation	Area	% Degradation	Area	% Degradation
Acid	190.078	19.835	193.057	18.579	186.274	23.3	189.389	22.017
Base	205.276	13.425	204.673	13.68	189.57	21.942	186.95	23.021
Thermal	188.234	20.613	184.765	22.076	163.553	32.655	162.639	33.032
Oxidation	203.616	14.126	184.765	22.076	165.6	31.812	167.695	30.95
Photo	161.942	31.701	161.186	32.02	175.273	27.829	173.045	28.747

IV. Conclusion

The Combined dosage form of Rivaroxaban and clopidogrel are not available commercially. But individually rivaroxaban is used as an anticoagulant and it is the first orally active direct factor Xa inhibitor and clopidogrel is used as an antiplatelet agent. Various methods are reported for the analysis of individual drug and in combination with other drugs but no stability indicating HPLC method reported for these two drugs in combined dosage form. Therefore, a novel RP- HPLC method has been developed for the simultaneous estimation of Rivaroxaban and Clopidogrelin combination. The optimized chromatogram was run for appropriate minutes with mobile phase Phosphate buffer (Ph 4.5): Methanol (70:30). Data related to peak like area, height, retention time, resolution etc. were recorded using software. Thermo scientific, C_{18}(25cm×0.46cm) Hypersil BDS, Mobile Phase Phosphate buffer, pH 4.5: Methanol (70:30) with Flow Rate 1.0 ml/min and Runtime 6 min Injection volume of 20.0 µl. The detection was carried out at wavelength 214 nm. It was found to be simple, precise and accurate. In this stability indicating RP-HPLC methods were developed by degradation of sample and compared with standard. The % RSD was also less than 2 % showing high degree of precision of the proposed method. The proposed method can be used for routine analysis of Rivaroxaban and Clopidogrel in combined dosage form. It can be also used in the quality control in bulk manufacturing.

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Evaluation of Methanolic Extract of Hypericum Mysorense Ointment for its Wound Healing Activity

By Sankar C, Muthukumar S, Arulkumaran G, Vinesha R, Manimekalaim & Sandeep George Simson

KMCH College of Pharmacy

Abstract- The main objective of the present study is to formulate and evaluate semi-solid dosage forms of *Hypericum mysorense* for its wound healing activity. The stem of *Hypericummysorense* was extracted by continuous hot percolation/ soxhletationusing methanol as solvent and was evaluated for its phytochemical property, *in-vitro* antimicrobial and*in-vitro* antioxidant activity. Using this methanolic extract, semi-solid dosage form(ointment) was formulated. Ointment was prepared using 2% concentration of the extract by fusion method using emulsifying ointment. Hence, the ointment containing 2% methanolic extract of *Hypericummy-sorense* was evaluated for its wound healing activity using excision wound model. The effect produced by the extract ointment, in terms of wound contracting ability, wound closure time and histopathological characteristics were comparable to that of a standard drug povidone iodine ointment. Therefore, the present study concluded that the *Hypericum mysorense* at 2% concentration of the methanolic extract formulated as an ointment showed a better wound healing formulation for better patient care and pharmaco economical.

Keywords: *hypericum mysorense, excision wound model, ointment.*

GJMR-B Classification: *NLMC Code: QV 55*



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Evaluation of Methanolic Extract of Hypericum Mysorense Ointment for its Wound Healing Activity

Sankar C [α], Muthukumar S [σ], Arulkumaran G [ρ], Vinesha R [ω], Manimekalaim [¥] & Sandeep George Simson [§]

Abstract- The main objective of the present study is to formulate and evaluate semi-solid dosage forms of *Hypericum mysorense* for its wound healing activity. The stem of *Hypericummysorense* was extracted by continuous hot percolation/ soxhletationusing methanol as solvent and was evaluated for its phytochemical property, *in-vitro* antimicrobial and*in-vitro* antioxidant activity. Using this methanolic extract, semi-solid dosage form(ointment) was formulated. Ointment was prepared using 2% concentration of the extract by fusion method using emulsifying ointment. Hence, the ointment containing 2% methanolic extract of *Hypericummy-sorense* was evaluated for its wound healing activity using excision wound model. The effect produced by the extract ointment, in terms of wound contracting ability, wound closure time and histopathological characteristics were comparable to that of a standard drug povidone iodine ointment. Therefore, the present study concluded that the *Hypericum mysorense* at 2% concentration of the methanolic extract formulated as an ointment showed a better wound healing formulation for better patient care and pharmaco economical.

Keywords: hypericum mysorense, excision wound model, ointment.

I. Introduction

a) Overview of Traditional Herbal Medicine

India is a birthplace of indigenous medicine such as Siddha, Ayurveda, and Unani where many herbs have been used for treatment of human ailments. About 65% of total global population remains dependent on traditional medicines for their primary healthcare. Herbs are occupying a comeback and an 'Herbal Renaissance' is blooming across the world. They have been evidently prized for their medicinal, flavoring and aromatic qualities for centuries, yet for a while they were over shadow by synthetic products of modern civilization. Folk medicine is generally defined as traditional medicine that is practiced by non-professional healers or embodied in local custom or lore, generally involving the use of natural and especially herbal remedies.

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Once having realized their sources and adverse effects, people are going back to nature with hopes of safety and security. The rich treasure of herbal drugs is forming a boon for our society. Plant derived compounds, apart from their nutritive values, could serve as important therapeutic weapons to fight various human and animal diseases, thereby making them indispensable in traditional medicine for treating a number of diseases. Plant drugs, popularly known as herbal medicines have since been unabatedly used to treat various diseases. The major challenge is to protect traditional knowledge and will prove to be a beneficial asset to our human surrounding. For all the ailments herbal formulations are proved to be effective without any side effects commonly seen with allopathic drugs.

II. Ointment

An ointment is a homogeneous, viscous, semi-solid preparation, most commonly greasy, thick oil (oil 80% - water 20%) with a high viscosity which is intended for external application to the skin or mucous membranes. Ointments have a water number that defines the maximum amount of water that it can contain. They are used as emollients or for the application of active ingredients to the skin for protective, therapeutic, or prophylactic purposes and where a degree of occlusion is desired. Ointments are used topically on a variety of body surfaces. These include the skin and the mucous membranes of the eye (an eye ointment), vagina ,anus, and nose. An ointment may or may not be medicated. Ointments are usually very moisturizing, and good for dry skin. They have a low risk of sensitization due to having few ingredients beyond the base oil or fat, and low irritation risk. There is typically little variability between brands of generics and name brand drugs. They are often disliked by patients due to greasiness.

III. Wound Healing Activity

Wounds are inescapable events in life. Wounds may arise due to physical, chemical or microbial agents. Wound healing involves a complex series of interactions between different cell types, cytokine mediators, and the extracellular matrix. The phases of normal wound

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healing include hemostasis, inflammation, proliferation and remodeling. Each phase of wound healing is distinct, although the wound healing process is continous, with each phase overlapping the next. Because successful wound healing requires adequate blood and nutrients to be supplied to the site of damaged tissue.

IV. Materials and Methods

a) Collection And Extraction

The leaves of Hypericum mysorense was collected freshly during the month of October from Ooty, India, identified and authenticated .The collected plant, Hypericum mysorense was extracted by continuous hot percolation (soxhletation). 200g of powdered stem of Hypericum mysorense was defatted using petroleum ether. The marc obtained from the powdered plant part was successfully extracted with 250 ml of methanol by using soxhlet apparatus. The extraction was carried out for 48 hours. After extraction, the solvents were distilled out; the concentrated residues were analyzed by chemical tests.

b) Preparation of Semi-Solid Formulation

i. *Formulation of Ointment*

Preparation of Ointment Base(Emulsifying Ointment)

Emulsifying wax - 50g,White soft paraffin - 20g, Liquid paraffin - 30g

Procedure

Required quantities of emulsifying wax, liquid paraffin and white soft paraffin were weighed and melted. To this, adequate quantity of methanolic extract of plant was added and stirred well until a homogeneous mass were obtained. The composition of the prepared herbal ointment (F1) is listed in Table I.

Table I: Composition of Ointment

Sl. No.	Ingredients	F1
1	Methanolic extract of *Hypericum mysorense* stem	2gm
2	Emulsifying Ointment	q.s to 100gm

c) Pharmacological Evaluation of the Formulated Ointment

i. *Acute Skin Irritation Study*

The primary skin irritation test was performed on albino rats and weighing about 150-200gm. The animals were maintained on standard animal feed and had free access to water *ad libitum*. The animals were kept under standard laboratory condition. The total mass was divided into four batches, each batch containing six animals. Two batches of each were used for control and test. Dorsal hairs at the back of the rats were clipped off one day prior to the commencement of the study. Animals showing normal skin texture were housed individually in cages with meshes to avoid contact with the bedding. 50mg of the each formulation of different concentrations were applied over one square centimeter area of intact and abraded skin to different animals. Aqueous solution of 0.8% formalin was applied as standard irritant. The animals were observed for seven days for any signs of oedema and erythema.

ii. *Wound Healing Activity*

a. *Animals*

Wister rat of male sex, weighing 150-200g were used. All animals were obtained from KMCH College of Pharmacy. All animals were housed for at least one week in the laboratory animal room prior to testing. The selected animals were housed in polypropylene cages in standard environmental conditions (20-25°c). Fed with standard rodent diet and water as libitum. The experiments on animals were conducted in accordance with the international accepted principles for laboratory animal use and the experimental protocols duly approved by the institutional ethical committee ,KMCH college of pharmacy, Coimbatore, Reg No. 685/Po/02/a/CPCSEA.Dt:21st Aug 2002, IAEC No: KMCRET/M.Pharm/9/2013-14

V. Results

The colour of the ointment was dark green with characteristic odour. The result clearly indicated that the ointment showed good spreadability and extrudability. The ointment showed a pH that lie in the normal pH range of human skin and iseasily diffusible.

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Pharmacological evaluation of the formulated ointment

a) Wound healing activity

Table 7: Percentage Wound closure

Test Compounds	0th day	3rd day	7th day	13th day	17th day	19th day
	% WC	% WC	% WC	% WC	%WC	%WC
STD	0	15.7 ±0.29	39.8±0.77	81.8±1.09	88.5±1.25	95.5±1.10
Ointment Base	0	13.5 ±0.49	27±0.83	52.5±1.24	66.8±1.24	88.2±1.88
Control	0	27.5±0.58	23.6±2.03	74.2±0.99	72±0.99	90.1±0.99
Ointment Containing 2%Extract	0	10±0.42	13.8±1.48	55.4±1.08	84.1±1.08	96.9±1.08

Results were expressed as mean (mm) ± SEM and were compared with the corresponding control group (simple ointment B.P.) by applying ANOVA test. P value was set <0.05 for all analyses.

Table 8: Figure showing percentage wound reduction

Test compounds	TH 0 day	3rd day	7th day	13th day	17th day	19th day
	%WR	%WR	%WR	%WR	%WR	%WR
STD	100±0.00	84.3±0.29	60.2±0.77	18.2±1.09	11.5±1.25	4.42±1.10
Ointment Base	100±0.00	86.5±0.49	73±0.83	47.5±1.24	33.2±1.24	11.8±1.88
Control	100±0.00	72.5±0.58	76.4±2.03	25.8±0.99	28±0.99	9.81±0.99
Ointment Containing 2% Extract	100±0.00	90±0.42	86.1±1.48	44.5±1.08	15.8±1.08	3.02±1.08

Results were expressed as mean (mm) ± SEM and were compared with the corresponding control group (simple ointment B.P.) by applying ANOVA test. P value was set <0.05 for all analyses.

Excision Wound On 0th Day





Only Wound Wound + Ointment Base





Wound + Standard

Wound +Oint Containing 2% Meoh.Hms

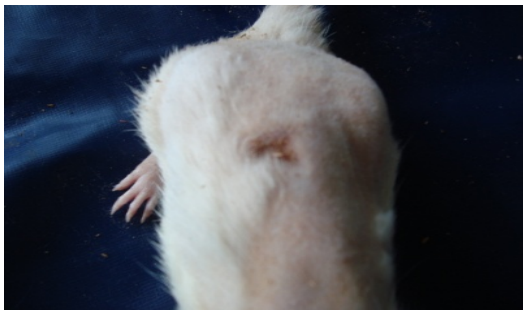
Figure: Figure showing the wound healing activity from 0thday — 21th

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Excision Wound on- 21st Day



Only Wound



Wound + Ointment Base



Wound + Oint Containing 2% Meoh.Hms



Wound+Standard

VI. Discussion

Hypericum mysorense is an ornamental plant belonging to the family Hypericaceae, having antibacterial activity, against both gram-positive and gram-negative bacteria. Leaf and flower have strong antioxidant potential and used for liver disorders. Its stem is having strong antitumor, antipsychic and antiviral activities. Among the various indications where traditional herbal medicines are used, skin and skin related disorders are ranked top. Thus, the main objective of the present study is to formulate and evaluate semi-solid dosage forms of Hypericum mysorense for its wound healing activity. The stem of Hypericum mysorense was extracted by continuous hot percolation/ soxhletation using methanol as solvent,Using this methanolic extract, semi-solid dosage form (ointment) was formulated. Ointment was prepared with 2% methanolic extract using emulsifying ointment base.Hence, the ointment containing 2% Methanolic Extract of Hypericum mysorense stem was evaluated for its wound healing activity using excision wound model. The formulation did not produce any skin irritation for about a week when applied over the skin. Wister rats weighing around 150-200g were used for the study. They were divided into 4 groups consisting of 6 rats each. The 4 groups include standard(povidone iodine ointment), control(only wound), rats treated with ointment base and rats treated with 2% ointment. The effect produced by the extract ointment, in terms of wound contracting ability, wound closure time and histopathological characteristics were comparable to that of a standard drug, povidone iodine ointment. Thereby, the ointment formulated using 2% methanolic extract of Hypericum mysorense was found to possess wound healing activity.

VII. Conclusion

The effect produced by the extract ointment, in terms of wound contracting ability, wound closure time and histopathological characteristics were comparable to that of a standard drug, povidone iodine ointment. Therefore, the present study concluded the wound healing activity of *Hypericum mysorense*stem at 2% concentration of the methanolic extract formulated as an ointment.

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Conflict of Interests

The authors declare that they have no conflicts of interest.

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Determinants of Hypertension in a Rural Area of Kancheepuram District, Tamilnadu

By Dr. M. Vijayakarthikeyan & Dr. Muthulakshmi Muthiah

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Abstract- *Background:* Hypertension is one of the most important modifiable risk factors for cardiovascular diseases (CVDs). Hypertension is a risk factor that accounts for 12.3% of the deaths and disabilities combined in Tamilnadu during 2016.

Objectives

- To assess the prevalence of risk factors of hypertension among the study population.
- To determine the association between socio-demographic factors and hypertension.
- To determine the association between various risk factors and hypertension.

Keywords: *blood pressure, risk factor, cardiovascular disease.*

GJMR-B Classification: *NLMC Code: WG 340*



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Determinants of Hypertension in a Rural Area of Kancheepuram District, Tamilnadu

Dr. M. Vijayakarthikeyan [α] & Dr. Muthulakshmi Muthiah [σ]

Abstract- Background: Hypertension is one of the most important modifiable risk factors for cardiovascular diseases (CVDs). Hypertension is a risk factor that accounts for 12.3% of the deaths and disabilities combined in Tamilnadu during 2016.

Objectives

- To assess the prevalence of risk factors of hypertension among the study population.
- To determine the association between socio-demographic factors and hypertension.
- To determine the association between various risk factors and hypertension.

Materials and methods: It is a community-based cross-sectional study. The sample size calculated was 1250 and a systematic random sampling method was used. A pre-tested structured questionnaire was used to collect data from the study population. Information regarding socio-demographic characteristics, risk factors, regarding hypertension and physical measurements were obtained. The data analysis was done using SPSS software (version 22).

Results: Among the study population,23.6% were hypertensive, and. in this study,12.8% use tobacco in any form, 18.6% use alcohol, 66.2% of the participants are physically inactive, and 72.5% are consuming an unhealthy diet. In the Univariate analysis, the variables that are significantly associated with hypertension are age, marital status, education, occupation, socio-economic status, family type, positive family history, presence of associated comorbidities, knowledge about hypertension and BMI. In multivariate analysis are age, presence of associated comorbidities, knowledge about hypertension, family type, and BMI.

Conclusion: The prevalence of hypertension and its determinants is high in this study are tumultuous. Lifestyle modification plays a pivotal role, and hypertension is a lifestyle disease change in that harmful lifestyle habits must be adopted.

Keywords: blood pressure, risk factor, cardiovascular disease.

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I. Introduction

High blood pressure (BP) is one of the most important modifiable risk factors for cardiovascular diseases (CVDs). [1]Hypertension (HTN) is a chronic condition of concern because of its role in the causation of coronary heart disease (CHD), stroke, and other vascular complications. It is the most common CVD disorder which poses a significant public health challenge to a population undergoing socioeconomic evolution. It is one of the dominant risk factors for CVD mortality, accounting for 20-50% of all deaths. [2,3]Hypertension (HTN) exerts a substantial public health burden on cardiovascular health status and healthcare systems in India.[4,5]

The analysis showed that about 26% of the population globally is suffering from hypertension, and the prevalence is higher among developed as compared to developing countries.[6] It is predicted that the number of adults with hypertension would increase by about 60% to a total of 1.56 billion by 2025.[7,8]HTN is directly responsible for 57% of all stroke deaths and 24% of all coronary heart disease (CHD) deaths in India.[9,10]

Currently, the incidence of hypertension is 20 to 40% in urban areas and 12 to 17% in rural areas of India. One in three Indian adults has high blood pressure. According to the World Health Statistics 2012 report, India has low rates of hypertension compared to world figures.[11]In India, 23.10% of men and 22.60% of women over 25 years suffer from hypertension.[12,13]As per the NFHS 4 report, prevalence of hypertension in males is 10.3% and in females is 6.7% .[14] Community surveys have documented that in a period of three to six decades, prevalence of hypertension has increased by about 30 times among the urban dwellers and by about ten times among the rural inhabitants.[15,16]

The technological and economic developments have reduced the physical activity of the people to a real large extent and increased the alcohol and tobacco use which are the vital causes for the rising burden of hypertension.[17]The risk factors for non-communicable disease are grouped into three categories they are behavioral, metabolic and biochemical risk factors. Behavioral risk factors include tobacco use, alcohol use, unhealthy diet, and lack of physical activity. Metabolic risk factors include overweight, obesity, diabetes, and

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hypertension (HTN). Biochemical risk factors include hypercholesteremia and hypertriglyceridemia.[18]

To contain the increasing burden of Non-Communicable Diseases, Ministry of Health and Family Welfare, Government of India, has launched the National Programme on Prevention and Control of Diabetes, Cardiovascular Diseases and Stroke (NPDCS).[19]As fewer studies has been undertaken in rural India, this study was planned to assess the determinants of hypertension among the rural population of Kancheepuram district of Tamil Nadu. This study will shed some light on the existing problem.

II. Materials and Methods

a) Study design

This study is a community-based cross-sectional study conducted in a rural area of Kancheepuram district, Tamil Nadu.

b) Study area

The study was conducted in Serappanachery Padappai (S. Padappai), which is the rural field practice area of the Rural Health and Training Centre (RHTC) attached to our Institution (Sree Balaji medical college and hospital).

c) Study population

The study population included are those permanently residing in Serappana-chery Padappai and belonging to the adult age group of 20–60 years.

d) Study period

The study was conducted during December 1st2018 –May 31st, 2019.

e) Sample size

The sample size was calculated from a previous study conducted by Kishore J et al, in a rural area in 2016, the prevalence of hypertension recorded in this study was 14.1%.[20] The sample size was calculated using the formula $N = Z\acute{\alpha}^2pq/ [L]^2$ where Z = 1.96, p = 14.1%, q =85.9 (100-14.1), L = 2. 115. Accounting 15% for non-response, the final sample size was calculated as 1245 (rounded off to 1250). [N = 1250]

f) Inclusion criteria

The inclusion criteria for the study were the adult population of age group (20-60 years) residing in Serappanachery Padappaiand willing to participate in the study.

g) Exclusion criteria

The exclusion criteria for the study were females who were pregnant, psychiatric patients, who are severely ill, and those who didn't give consent to participate in the study was excluded.

h) Sampling method

A systematic random sampling technique was used to identify the study subjects. Sampling Interval (N/n) is calculated as follows: [N= Total number of households in Padappai=1851, n = sample size = 1250. N/n=1851/1250= 2]. Thus alternate household is selected for identifying the adult population between 20-60 years of age.

i) Study tool

A structured questionnaire based on the WHO STEPS approach is used as a study stool for data collection, Details included in it are socio demographic profiles, details regarding risk factors for hypertension, and physical measurements (height, weight, waist circumference, and BP).

j) Informed consent

Informed Consent was obtained from each participant before the administration of the interview schedule.

k) Ethical approval

The study proposal was presented and was approved by the Institutional Ethics Committee.

l) Operational definitions

1. Tobacco user:[21]
   Tobacco user was defined as individuals who had used any form of tobacco in the last 30 days.
2. Alcohol user:[21]
   Alcohol users were those who had consumed at least one standard drink of alcohol (30 ml of spirits, 285 ml of beer, or 120 ml of wine) in the last 12 months.
3. Unhealthy diet:[18]
   A unhealthy diet is Low consumption of fruits and vegetables at less than five servings per day (one cup of raw leafy vegetables or a half cup of other vegetables (cooked) was considered one serving. One medium-sized piece of fruit or half cup of chopped fruit was measured as one serving).
4. Physical activity:[18]
   Physical activity low physical activity was defined as <150 minutes of moderate physical activity per week.
5. Overweight:[22]
   Overweight was defined as BMI 23-24.9 kg /m2.
6. Pre obese:[22]
   Pre obese was defined as BMI equal to or more than 25 kg /m2.
7. Obese:[22]
   Obese was defined as BMI equal to as or more than 30 kg /m2.
8. Central obesity:[22]
   Central obesity is assessed based on the waist-hip ratio. As per WHO guideline, males with a waist-hip ratio above 0.9 and females with a waist-hip ratio above 0.85 have central obesity.

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III. Results

a) Socio-demographic characteristics of the study population

Socio-demographic characteristics of the study population are shown in Table 1. Among the study participants, 44.2% belonged to 50-60 years of age, 24.2% belonged to 20-30 years of age, and 20.8% belonged to 30-40 years of age. About 57.4% of the study participants were females, and 42.6% were males. Nearly 82.4% are married, and 5.44% were unmarried. Almost 18.7% of the study samples had no formal education, 30.2% had middle school education, and 21.3% had a high school education. Among the participants, around 43.7% were unemployed, 32.2% are engaged in unskilled occupation, and 17.8% are involved in semiskilled occupation.49.6% belonged to lower- middle socio-economic category, and 21.8% belonged to the upper lower socio-economic group. In this study, 56.8% of them belong to the nuclear family,30% belonged to the joint family, and the rest were belonging to three-generation family.

Table 1: Socio Demographic Characteristics of the Study Population

Sl. No.	Socio-Demographic Variable	Frequency (N=1250)	Percentage (%)
1.	**Age**		
	20-30 Years	136	10.9
	30-40 Years	302	24.2
	40-50 Years	260	20.8
	50-60 Years	552	44.2
2.	Sex		
	Male	532	42.6
	Female	718	57.4
3.	**Marital Status**		
	Unmarried	68	5.4
	Married	1030	82.4
	Widower	142	11.4
	Divorcee	10	.8
4.	**Education**		
	Illetereate	234	18.7
	Primary School	282	22.6
	Middle School	378	30.2
	High School	266	21.3
	Post High School Diploma	12	1.0
	Ug/Pg	72	5.8
	Professional	6	.5
5.	**Occupation**		
	Unemployed	546	43.7
	Unskilled	402	32.2
	Semiskilled	222	17.8
	Skilled	46	3.7
	Farmers/Clerks/Shop Owners	10	.8
	Semiprofessional	14	1.1
	Professional	10	.8

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6.	Socio Economic Status		
	Upper	90	7.2
	Upper Middle	234	18.7
	Lower Middle	620	49.6
	Upper Lower	272	21.8
	Lower	34	2.7
7.	TYPE OF FAMILY		
	Nuclear Family	710	56.8
	Joint Family	438	35.0
	Three Generation Family	102	8.2

b) Prevalence of hypertension

The prevalence of hypertension is depicted in FIGURE 1.as we can see, the prevalence of hypertension in this study is 23.8% (298). This includes both known hypertensives and newly diagnosed.

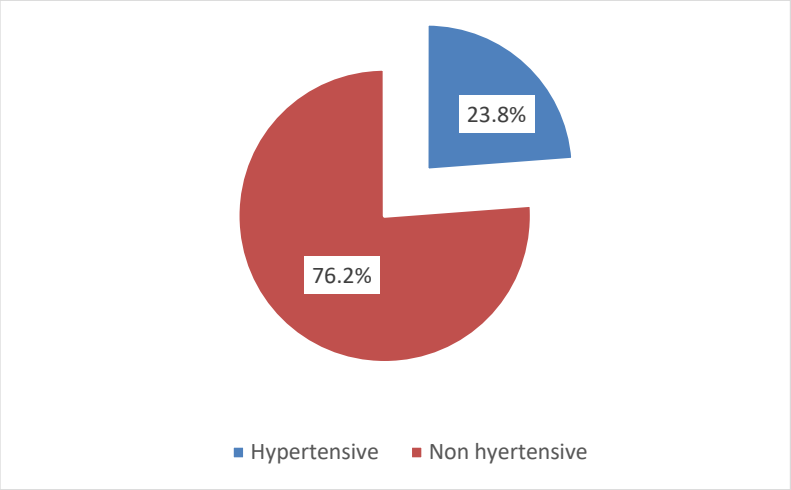


Fig. 1: Food habits of the study population

As we can see from TABLE 2, nearly 89.9% of the study participants were nonvegetarian, and 10.1% were vegetarian. Among the non-vegetarians, 54.1% had nonvegetarian once a week, and 35.1 had nonvegetarian twice a week.

Table 2: Food Habits of the Study Population

Sl. No.	Food Habits	FREQUENCY	PERCENTAGE (%)
1.	Food Type (N-1250)		
	Vegetarian	126	10.1
	Non Vegetarian	1124	89.9
2.	Frequency of Non-Veg Intake (N-1124)		
	Once A Week	608	54.1
	Twice A Week	394	35.1
	Thrice A Week	104	9.2
	Four Times A Week	12	1.1
	> Four Times A Week	6	0.5

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c) Lifestyle characteristics of the study population

Lifestyle characteristics of the study population are shown in TABLE 3.60.2% of them are involved in sedentary work, and 35.5% were engaged in the moderate type of work. Only 21.4% have the habit of doing regular physical exercise. Among them, 39.6% do it for 1-2 hours per week and 27.7% do it for 2-5 hours a week.

Table 3: Lifestyle Characteristics of the Study Population

Sl. No.	Lifestyle	FREQUENCY	PERCENTAGE (%)
1.	Job Type (N-1250)		
	Sedentary Work	752	60.2
	Moderate Work	444	35.5
	Heavy Work	54	4.3
2.	Exercise (N-1250)		
	Yes	268	21.4
	No	982	78.6
3.	Duration of Exercise (N-268)		
	< 1 Hour/Week	20	7.4
	1-2 Hours/ Week	106	39.6
	2--5 Hours/ Week	74	27.7
	> 5 Hours/ Week	68	25.3

Table 4: Prevalence of Behavioural Risk Factors Among the Study Population

Sl. No.	Risk Factor	Frequency (N-1250)	Percentage (%)
1.	Tobacco Use		
	Yes	160	12.8
	No	1090	87.2
2.	Alcohol Use		
	Yes	232	18.6
	No	1018	81.4
3.	Physical Inactivity		
	Yes	828	66.2
	No	422	33.8
4.	Unhealthy Diet		
	Yes	906	72.5
	No	344	27.5

d) Prevalence of risk factors among the study population

Prevalence of risk factors for hypertension is depicted in TABLE 4 and 5.in this study 12.8% use tobacco in any form, 18.6% use alcohol, 66.2% of the participants are physically inactive, and 72.5% are consuming an unhealthy diet. Among the study participants, 21.4 % had a positive family history of hypertension,3% of them are under oral contraceptive pills, and 24.8% are suffering from various comorbidties (TABLE 5).

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Table 5: Associated Risk Factors among Study Population

Sl. No.	Risk Factors	Frequency (N-1250)	Percentage (%)
1.	Family History of Hypertension (N-1250)		
	YES	268	21.4
	NO	982	78.6
2.	OCP Pill Intake Among Females (N-718)		
	YES	38	3.0
	NO	680	97.0
3.	Co-Morbidity (N-1250)		
	YES	310	24.8
	NO	940	75.2

e) Prevalence of obesity among the study population

As per the Asian Adults BMI criteria (FIGURE 2), 26.2% were overweight, 22.% were pre-obese and 12.6% belonged to the obese category. Central obesity was assessed based on the waist-hip ratio. About 83.4% of the study participants have central obesity.

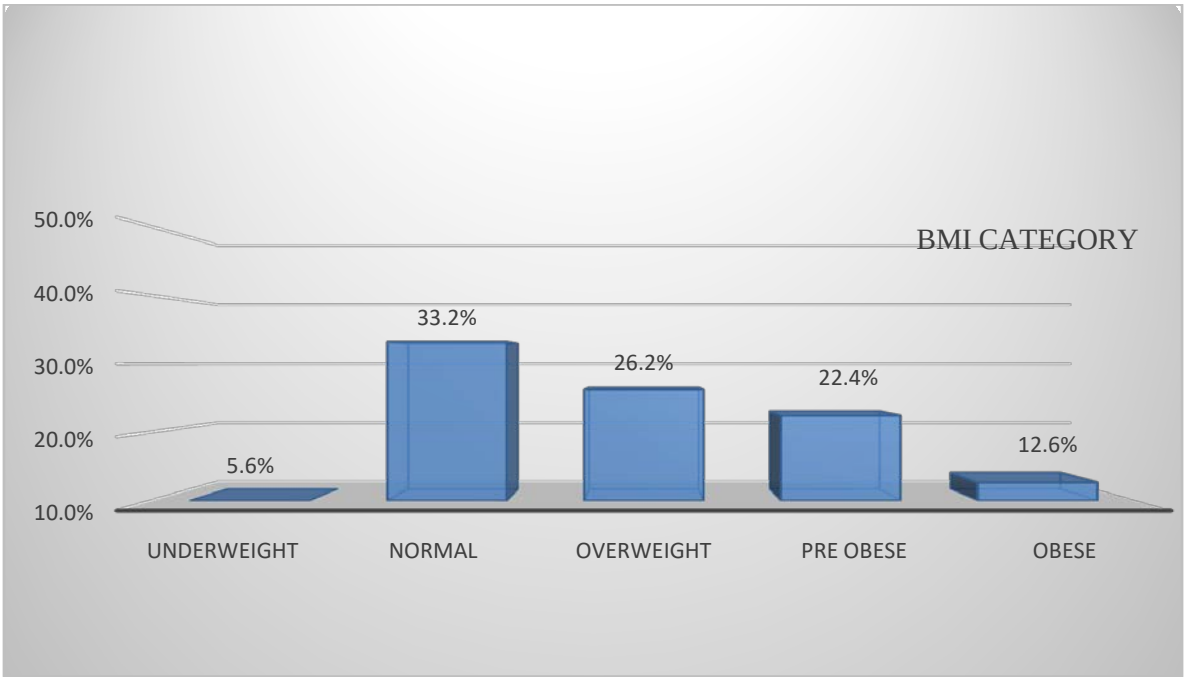


Figure 2: Bmi Classification of the Study Population

Among the males, 86.5% have central obesity, and 80.3% of females have central obesity in this study (FIGURE 3).

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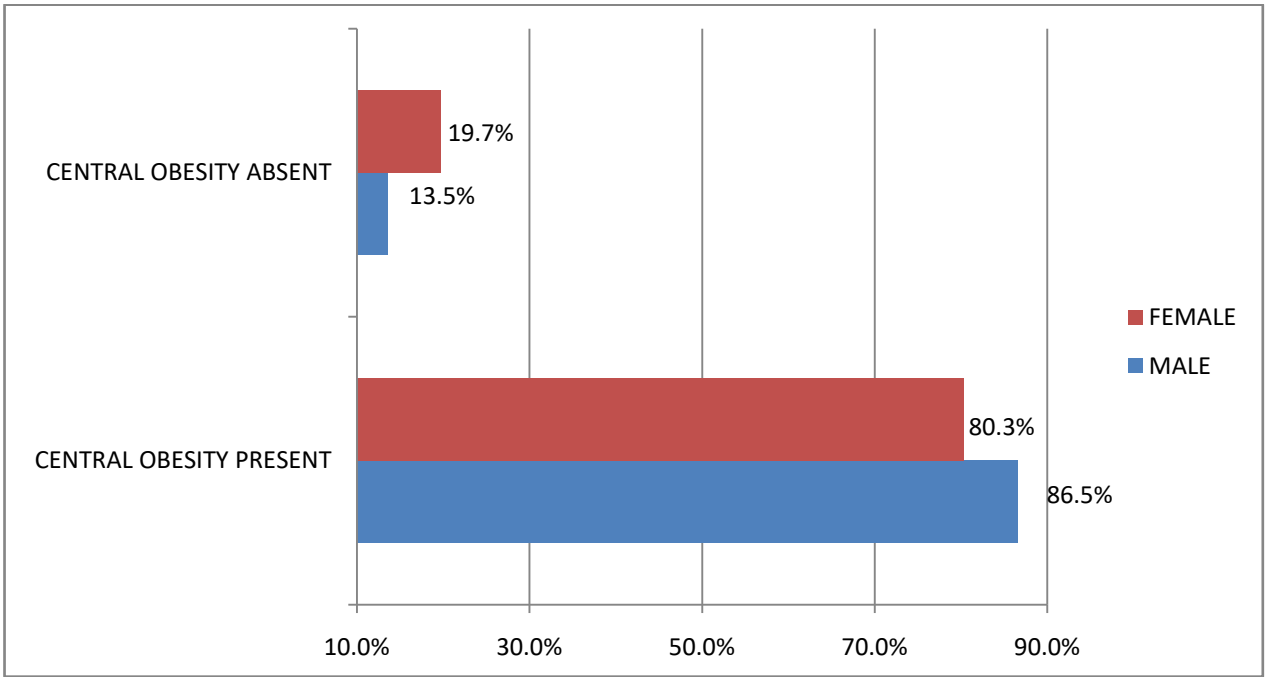


Figure 3: Prevalence of Central Obesity among the Study Population

f) Knowledge regarding hypertension among the study population

Among the study participants, when asked whether they know the normal blood pressure value, 24.8% said they know the normal blood pressure value., and among them, only 60% said the correct blood pressure value and 40% said incorrect value. In this study, 47.5% of the participants have adequate knowledge about hypertension, as shown in FIGURE 4.

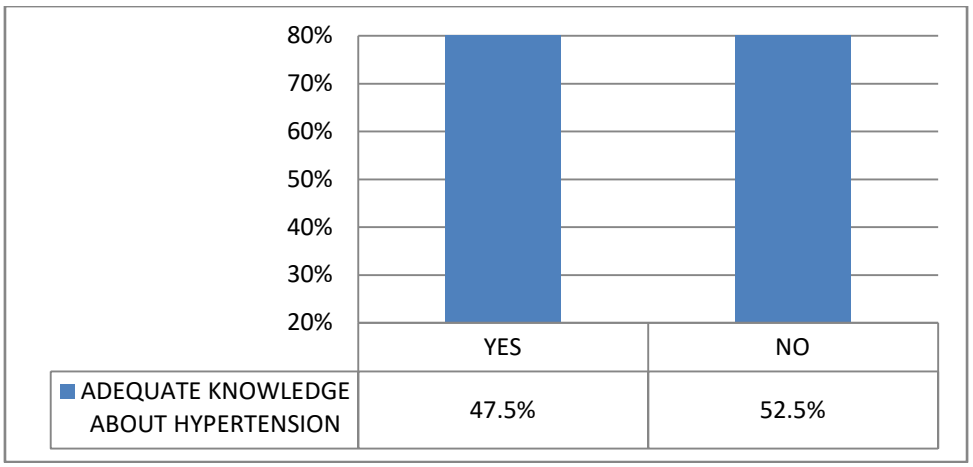


Figure 4: Adequate Knowledge Regarding Hypertension Among The Study Population

g) Univariate analysis findings among the study population

In the Univariate analysis the variables that are significantly associated with hypertension are age (p-value-<0.0001), marital status (p-value-<0.0001), education (p-value-0.015), occupation (p-value-0.003), socio-economic status (p-value-<0.015), family type (p-value-<0.0001), positive family history (p-value-0.009), presence of associated comorbidity (p-value-<0.0001), knowledge about hypertension (p-value-<0.0001) and BMI (p-value-<0.0001). There was no association found between other variables and hypertension.

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Table 7: Univariate Analysis Findings

Variable	Total Frequency (N-1250)	Hypertension			
		FREQUENCY (N-298)	CHI-SQUARE VALUE	P VALUE	ODDS RATIO (95%CI)
Age > 40 Years < 40 Years	 438 812	 264 34	124.387	<0.0001***	5.724 3.916-8.366
Sex Female Male	 532 718	 168 130	0.181	0.670	0.949 0.730-1.234
Marital Status Married Unmarried/Divorce /Widower	 1030 120	 226 72	23.943	<0.0001***	17.051 12.407-23.434
Education ≥ High School Education < High School Education	 356 894	 91 207	15.828	0.015**	1.139 0.857-1.514
Occupation ≥ Skilled < Skilled	 80 1120	 22 276	19.501	0.003***	1.225 0.736-2.039
Socioeconomic Status Upper / Middle Class Lower Class	 944 306	 227 71	12.295	0.015**	1.047 0.772-1.420
Family Type Joined/Three Generation Family Nuclear Family	 540 710	 158 140	22.244	<0.0001***	1.684 1.296-2.188
Tobacco Use Yes No	 160 1090	 33 265	1.045	0.307	0.808 0.538-1.125
Alcohol Use Yes No	 232 1018	 59 239	0.397	0.529	1.111 0.799-1.544
Unhealthy Diet Yes No	 906 344	 221 77	0.554	0.457	1.187 0.832-1.503
Physical Inactivity Yes No	 828 422	 194 104	3.427	0.180	0.940 0.715-1.235
Positive Family History Yes No	 268 982	 80 218	6.788	0.009***	1.491 1.102-2.016
Presence of Associated Comorbidity Yes No	 310 940	 129 169	71.718	<0.0001***	3.251 2.456-4.304
Knowledge About Hypertension Yes No	 570 680	 204 94	58.774	<0.0001***	3.071 2.329-4.050
Bmi Overweight/Preobese/Obese Underweight /Normal	 765 485	 234 64	20.277	<0.0001***	2.886 2.128-3.914

*** P value <0.05 is significant and *** P value <0.01 is highly significant*

h) Multivariate analysis findings among the study population

The variables which were significantly associated in Univariate analysis were only included in the multivariate analysis which is shown in TABLE 8. The multivariate analysis was done using the Enter method. The Model was found to be statistically significant (Cox and Snell R2 – 0.240, Nagelkerke R2 –0.3660, P-value <0.001). The variables that are significant in multivariate analysis are age, presence of associated comorbidity, family type, and BMI,Other variables were found to be insignificant in multivariate analysis.

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Table 8: Multivariate Analysis Findings

Variable	Hypertension			
	P Value	Adjusted Or	95% Ci	Nagelkerke R Square Value
Age	<0.0001	0.417	0.341-0.510	0.360
Marital Status	0.235	0.807	0.567-1.149	
Education	0.266	0.925	0.806-1.061	
Occupation	0.397	0.935	0.393-1.093	
Socio Economic Status	0.556	1.058	0.877-1.276	
Positive Family History	0.117	1.343	0.929-01.944	Cox And Snell R Square Value
Presence of Associated Comorbidity	<0.0001	2.516	1.806-3.505	
Knowledge About Hypertension	<0.0001	2.712	1.958-3.756	0.240
Bmi	<0.0001	0.530	0.459-0.611	
Family Type	0.0001	0.656	0.517-0.832	

*** P value < 0.05 is significant and *** P value < 0.01 is highly significant*

IV. Discussion

a) Risk factors for hypertension

i. *Tobacco use*

In this study among the study participants, 12.8% use tobacco and of which 3.5% use smokeless tobacco. In a study, by Chataut J, 40.2% of the study population has smoking habit. [23]25.5% of the ever used tobacco in a study by Maroof KA In Uttar Pradesh.[24] In Peter Lloyd-Sherlock study 64.6% had never smoked and 24.1% are smoking daily.[25] 15.9 % are smoking daily, and 73.2% are using smokeless tobacco in a study by Aroor Bhagyalaxmi which was conducted in a rural area of Gujarat, India.[26] Sathish Kumar conducted a study in Salem in which 24.7% had never used tobacco, and 25% are past users. [21]

ii. *Alcohol use*

In this stud, 18.6% are current alcohol users, and 1.1% were past users of alcohol. Sathish Kumar's study showed that 58.3% are using alcohol daily or a few days a week, and 28.6% had used alcohol in the past.[21] 40.9% are consuming alcohol in a study by Chataut J.[23] In a study by K. A. Maroof,35.5% had ever used alcohol,, and the remaining 64.5% had never used alcohol.[24] 76.8% had never consumed alcohol in their lifetime in a study conducted by Peter Lloyd-Sherlock.[25]

iii. *Physical activity*

In this study, 66.2% were physically inactive, and only 33.8% were physically active as per the operational definition, and this showed that the majority of the study participants are following unhealthy lifestyle habits. In Chataut J study, 51.8% are involved in moderate physical activity, and 8% are engaged in sedentary activities.[23] 28.5% are physically inactive in a study done by Peter Lloyd-Sherlock.[25] Aroor Bhagyalaxmi study showed that 14.1% of the study samples were physically inactive.[26] 34.9% were doing sedentary physical activity and 33.8% are involved in vigorous physical activities in a study done by Sathish Kumar.[21]

iv. *Unhealthy diet*

72.5% of the respondents in this study were following an unhealthy diet. In a study conducted by Aroor Bhagyalaxmi most of the study participants i.e. 96.4% were following unhealthy diet.[26] 94.5% were taking low fruit, and vegetables in a study by Garg A.[27]Bhattacherjee S conducted a study in West Bengal in which 60.4% were consuming an unhealthy diet.[28]

v. *Overweight and obesity*

In this study, as per the Asian Adults BMI criteria, 26.2% were overweight, 22 % were pre-obese, and 12.6% belonged to obese category. In V Mohan study 22.5% were overweight and 28.5% of the respondents are obese.[29]12% of the respondents were overweight in a study by Aroor Bhagyalaxmi.[26] In a study done by Prabhakaran D 35% of them were overweight, and 3.3% of the study participants belonged to theobese category.[30]20.5% were overweight, and 4.2% were the obese in Midha T.[31]

vi. *Central obesity*

In this study, central obesity was assessed based on the waist-hip ratio. About 83.4% of the study participants have central obesity. In a study by Isezuo SA. 13% of the study participants had central obesity.[32] Aroor Bhagyalaxmi showed that central obesity was present in 38% of the samples.[26]15.7% of the study participants have central obesity in AK Agarwal study.[33] In a study by K. A. Maroof 30.5% were centrally obese.[24]49.1% have central obesity in a study by V Mohan.[29]

vii. *Food habits*

In this study, nearly 89.9% of the study participants were nonvegetarian and 10.1% were vegetarian. Among the non vegetarians, 54.1% had non vegetarian food once a week, and 35.1 had non vegetarian food twice a week. In Chataut J study, 91.3% of them are were nonvegetarian and 8.7% were vegetarian.[23] Ina study by K. A. Maroof, 86.6% were vegetarian, and 13.4% were nonvegetarian.[24]28.6% were

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vegetarian and 71.4% were nonvegetarian in a study by Sathish Kumar.[21]

viii. *Family history of hypertension*

In this study, among the study participants, 21.4 % had a positive family history of hypertension. Rajeev Bhardwaj conducted a study in which only 4% of the study participants have a positive family history of hypertension.[34] In a study by Shyamal Kumar Das, 2.4% of the study participants had a positive family history of hypertension.[35]53.8% of the families have hypertension in a study by Haresh Chandwani.[37]

b) Association between sociodemographic variables, risk factors and hypertension

In this study in the Univariate analysis, the variables that are significantly associated with hypertension are age, marital status, education, occupation, socio-economic status, family type, positive family history, presence of associated comorbidity, knowledge about hypertension, and BMI. The variables that are significant in multivariate analysis are age, presence of associated comorbidity, knowledge about hypertension, family type, and BMI. In a study by Sathish Kumar, increasing age, male gender, increasing BMI levels, tobacco, alcohol, WHR were found to be significant independent predictors of hypertension and on multivariate analysis of these significant variables age, male gender, increasing BMI levels, were found to be significant after adjusting for other variables.[21] In hypertension study group multicentric study multiple logistic regression analyses identified a higher body mass index, higher education status, and prevalent diabetes mellitus as important correlates of the prevalence of hypertension.[37] Jonas JB conducted a study, in which hypertension was associated with higher age, higher body mass index, body height, Higher blood hemoglobin levels, and elevated blood urea concentration.[38]

V. Conclusion

The prevalence of hypertension and its determinants is high in this study are tumultuous. Lifestyle modification plays a pivotal role, and hypertension is a lifestyle disease change in that harmful lifestyle habits must be adopted. The target population for this strategy will be adolescents, and early adults, as the prevention of risk factors will curb the rates of hypertension and its risk factors.

This study will initiate an internalization process of the government sector to make it more attractive, viable, and reliable, thereby giving scope proper screening, early diagnosis and treatment, and to provide accessible quality tertiary care.

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Keywords: *arthritis, phytochemical, pepper.*

GJMR-B Classification: *NLMC Code: WB 925*



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Preliminary Phytochemical Analysis of a Polyherbal Siddha Formulation Milagu Leghium (Ml)

Subathra D [α] & Kirubakaran R [σ]

Abstract- Legiyam is one of the sublime formulations of internal medicine in the Siddha system. Milagu legiyam is a polyherbal formulation that is indicated for vatha disease. athmaratchamirtham is the literature evidence for this drug. The purpose of this paper is to prove the ML contains the bioactive components which were used for the above indication.

Keywords: *arthritis, phytochemical, pepper.*

I. Introduction

In siddha arthritis is being compared to vatha disease. Arthritis is one of the devastating illnesses which generally affect the people in the prevailing situation. So the management and treatment of this disease are important. In Siddha therapeutics are classified into internal & external. legiyam is one of the internal medicine. A Siddha literature athmaratchamirtham specifies a polyherbal formulation milagu legiyam for vatha disease. Hence we have selected this polyherbal formulation to evaluate the Phytochemical analysis to conclude the bioactive components which have the synergic anti-inflammatory & antioxidant activity.

II. Materials and Methods

a) Source and authentication of raw drugs

The required drugs for preparations of ML are purchased from an authorized center. Drugs are identified and authenticated by the Medicinal Botanist of Govt. Siddha Medical College, Palayamkottai, then they are purified, and the medicine was prepared at the P.G.Gunapadam Practical hall of Govt. Siddha Medical College, Palayamkottai.

Ingredients

Sl. No.	Drugs	Botanical Name	Part Used	Amount
1	Milagu	Piper nigrum	Seed	100palam 3500grams)
2	Akirakaram	Anacyclus pyrethrum	Root	1 palam (35 rams)
3	Seeragam	Cuminum Cyminum	Seed	1 palam 35grams)
4	Kirambu	Syzygium aromaticum	Flower	1 palam (35 rams)
5	Vaividangam	Emblica ribes	Seed	1 palam 35grams)
6	Aelam	Eletaria cardamomum	Unriped fruit	1 palam (35grams)
7	Kostam	Costus speciosus	Root	1 palam(35 grams)
8	Atimaduram	Glycyrrhiza glabra	Root	1 palam(35 grams)
9	Paththiri	Myristica fragrans		1 palam(35grams)
10	Narukkumoolam	Piper longum	Root	1 palam(35grams)
11	Karkandu	___	__	10palam(350gram)

b) Purification of raw drugs

Milagu: Soak in butter milk for three days. Then fry in the clay plate.
Akirakaram: Remove the adulterant and make it dry on the shade light.
Seeragam: Soak in Ca $(OH)_2$ water on 21 hours then dried in sun light.
Kirambu: Remove the adulterant and fry it.
vaividangam: Remove the adulterant and make it dry on the shade light
Aelam: Remove the adulterant and fry it
Thippli: Remove the adulterant and fry it
Omam: Soak in Ca $(OH)_2$ water on 3 hours then sun dried
Kostam: Just remove the adulterant and make it dry on the shade light
Thippili moolam: Just remove the adulterant and make it dry on the sun light.
Jathipathiri: Just remove the adulterant and make it dry on the shade light.

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c) Method of preparation

Take the above raw drugs are powdered separately except pepper, mixed all together. Pepper is crushed and added to 1 Thooni (21.5Lit.) of water and make a Decoction (1:8), ten Palam (350g) of Rock sugar is added to the above decoction and make the sugar solution. Add the powdered raw drugs to the sugar solution stir well. Add 1padi (1400 ml) Ghee and stir well until it reaches the required consistency. Add ½ Padi (700 ml) honey and blend well. Store it in a separate dry airtight container.

III. Determination of Extractable Method

Method Determination of water- soluble extraction

5gm of the ML macerated with100 ml distilled water in a closed flask for 24 hrs, shaken frequently for the first six hours in the shaker, and allowed to stand for the next 18 hrs. The macerate should be filtered rapidly, taking precautions against loss of solvent.25 ml of this filtrate is evaporated to dryness in a flat bottom shallow dish and dried at 105 Celsius and collected the extract is used for the phytochemical analysis.

IV. Procedure

Qualitative Result:

a) Test for Carbohydrates

Benedict's test: To 0.5 ml of test drug, about 0.5 ml of Benedict's reagent was added to it. The mixture was heated in a boiling water bath for 2 minutes. A characteristic colourd precipitate indicates the presence of sugar.

b) Glycosides

Keller-Killiani Test: 2 ml of the extract, glacial acetic acid, one drop 5% $FeCl_3$ and concentrated H_2SO_4 was added. The reddish brown colour appeared at the junction of two liquid layers, and the upper layer turned bluish- green indicating the presence of glycosides.

c) Steroids

Salkowski Test: 2 ml of extract, 2 ml of chloroform, and 2 ml of concentrated. H_2SO_4 was added. The solution was shaken by well. As a result chloroform layer turned red and acid layer shows greenish-yellow fluorescence.

d) Alkaloids

The extract was evaporated at a test tube. To the residue, dilute HCL was added, shaken well, and filtered.

e) Flavonoids

Shinoda Test: To the extract, 5 ml of 95% ethanol and few drops of concentrated hydrochloric acid was added into it. To this solution 0.5 gm of magnesium turnings were added. Pink coloration indicated the presence of flavonoids.

f) Tannins

Mayer's Test: To the 2-3 ml of filtrate Mayer's reagent was added. The formation of yellow precipitate showed the presence of tannins.

g) Lead Acetate Test

On the addition of lead acetate solution to the extract, white precipitate appeared.

h) Saponin

Foam Test: Drug extract was shaken vigorously with water. No persistent foam was formed.

i) Protein

Biuret Test: With 3 ml of the test solution, a few drops of 4% NaOH and 1% CuSO4 solution were added. The tubes were observed for violet or pink color formation.

j) Phenol

Ferric chloride Test: The extract was diluted to 5 ml with distilled water. To that a few drop of neutral 5% ferric chloride solution was added. A dark green colour indicates the presence of phenolic compounds.

k) Glycosides

0.5 mg of extract was dissolved in 1 ml of water, and then aqueous NaOH solution was added. The formation of yellow color indicates the presence of glycosides.

l) Triterpenoids

To the test solution, 2ml chloroform was added with few drops of concentrated Sulphuric acid (3ml) at the side of the test tube. An interface with a reddish-brown coloration was formed if terpenoids constituent is present.

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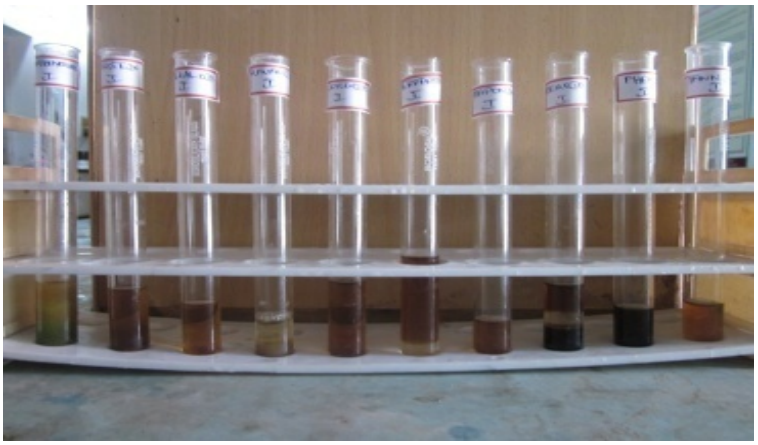


Image 1: Result of preliminary phytochemistry of ML

Table 1: Qualitative Result

Test Name	ML
Carbohydrate	Present
Protein	Absent
Alkaloid	Present
Flavanoid	Present
Glycoside	Absent
Steroid	Absent
Saponin	Absent
Phenol	Present
Tannin	Absent
Terpenoid	Absent

V. Quantitative Result

a) Procedure

i. *Quantitative Estimation of Alkaloids*

1ml of Methanolic extract 5 ml pH 4.7 phosphate Buffer was being added and 5 ml BCG solution and shaken a mixture with 4 ml of chloroform. The extracts have been collected in a 10-ml volumetric flask and then diluted to adjust volume with chloroform. The absorbance of the complex in chloroform was been measured at 470 nm against blank prepared as above but without extract. Atropine was been used as a standard material and compared the assay with Atropine equivalents.

ii. *Quantitative Estimation of carbohydrate*

Anthrone method (Roe, 1955) was used to estimate the total sugar content. A known amount of the sample have been taken, ground well with 80% ethanol and it was centrifuged at 4000 rpm. From the supernatant, 0.5 ml was taken and 5 ml of the anthrone reagent was added. The tubes were kept in a boiling water bath for 15 min. After that, they were kept in the darkroom for another 15 minutes. The color intensity developed was read in a spectrophotometer at 650 nm.

iii. *Quantitative Estimation of flavonoids*

Aluminium chloride method using catechin as a standard was taken as a method to determine the total flavonoid content.1ml of the test sample and 4 ml of water were added to a volumetric flask (10 ml volume). After 5 min 0.3 ml of 5 % Sodium nitrite, 0.3 ml of 10% Aluminium chloride was added. After 6 min it have been kept for incubation at room temperature, 2 ml of 1 M Sodium hydroxide was added to the reaction mixture. Immediately the final volume was been made up to 10 ml with distilled water. The absorbance of the reaction mixture was been measured at 510 nm against a blank spectrophotometrically. Results were been expressed as catechin equivalents (mg catechin/g dried extract).

iv. *Quantitative Estimation of Saponins*

Methanolic and water extract was dissolved in 80% methanol, 2ml of vanillin in ethanol was added, mixed well and the 2ml of 72% sulphuric acid solution was added, mixed well and heated on a water bath at 60^0c for 10min, absorbance was measured at 544nm against reagent blank. The Diosgeninis used as a standard material and compared the assay with Diosgenin equivalents.

v. *Quantitative Estimation of Phenolic Compounds*

The total phenolics content in different solvent extracts was determined with the Folin-Ciocalteu's reagent (FCR). In the procedure, different concentrations of the extracts were mixed with 0.4 ml FCR (diluted 1:10 v/v). After 5 min, 4 ml of a sodium carbonate solution was added. The final volume of the

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tubes was made up to 10 ml with distilled water and allowed to stand for 90 min at room temperature. The absorbance of the sample was measured against the blank at 750 nm using a spectrophotometer. A calibration curve was constructed using Gallic acid solutions as standard (0 to 250$\mu g/\mu l$).

vi. *Total terpenoid determination*

Total terpenoid content was being determined by the method of Ghorai et al, (2012). To 1 mL of the plant extract, 3 ml of chloroform was added. The sample mixture was thoroughly vortex and left for 3 min, and then 200 µl of concentrated sulfuric acid (H2SO4) was added. Then it was being incubated at room temperature for 1.5h-2h in dark condition and during incubation, a reddish-brown precipitate was formed. Then carefully and gently, all supernatant of the reaction mixture was decanted without disturbing the precipitation. 3 ml of 95% (v/v) methanol was added and www.ijppr.humanjournals.com Citation: Natesan Geetha et al. Ijppr. Human, 2015; Vol. 2 (2): 98-106. One hundred vortex thoroughly until all the precipitation dissolves in methanol completely. The absorbance was read at 538 nm using a UV/visible spectrophotometer. The total terpenoid content was calculated by calibration curve of Linalool and the results were expressed as Linalool equivalent (mg/g)

Table 2: Quantitative result

Phytochemical	Quantity
Carbohydrate	90±0.09
Alkaloid	74±0.41
Flavanoid	42±0.28
Phenol	60±0.29

VI. Result

The preliminary phytochemistry of the polyherbal formulation ML (aqueous solvent) is done by standard procedure. The results of the qualitative and quantitative bioactive components are presented in Table1 and Table2, respectively. The study discloses Carbohydrate, Alkaloid, Flavonoid, Phenol are the bioactive component present in the ML.

VII. Discussion

ML is a polyherbal formulation that has indicated for vatha disease in Siddha. The bioactive component present in this formulation poses the activity which was antioxidant and anti-inflammatory. ML containing biochemicals phenol and flavonoid are primary antioxidants. In the human body they prevent the body from the harmful effects of free radicals. Alkaloid poses many medicinal uses that may be having an anti-inflammatory action. Further evolution for isolate the particular alkaloid is needed.

VIII. Conclusion

Antioxidants are very potent biological active components which are participating in detoxification, cell repair of the body. Otherwise, one most important & all peoples in our world likes to maintaining our body as younger every day these antioxidants can retain your age by preventing aging. It also strongly reveals a Siddha formulation ML treats and prevents degenerative disorders like age-related arthritis. This phytochemical screening study accepted a remarkable scope to develop a broad use of Siddha medicine and as a base for further researches.

Acknowledgement

We are first of all, thanks to our parents for supporting this Study. And then thanks to scientific officers in Inbiotic Laboratory for analysis of this Drug ML. And finally, I thank all my friends who have helped this study.

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In-Vitro Anti-Microbial and Anti-Urolithiatic Models for Extract of *Parmelia Perlata* Lichen: An Evaluation of Prophylactic Management Against Kidney Stone

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Abstract- *Background:* Phyto-medicine is regaining interest owing to its advantages over conventional drugs and increasing cases of drug resistance. Moreover, recurrence and persistent side effects of present-day treatment for urolithiasis restrict their use, so an alternate solution using phytotherapy is being sought. The lichen species *Parmelia Perlata (*family *Parmeliaceae)* mentioned in India Materia Medica are useful in treating several ailments, and they are being used in large quantities as a food supplements in India.

Keywords: *parmelia perlata, lichen, phytochemicals, antimicrobial, anti-urolithiatic.*

GJMR-B Classification: *NLMC Code: WS 135*



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In-Vitro Anti-Microbial and Anti-Urolithiatic Models for Extract of *Parmelia Perlata* Lichen: An Evaluation of Prophylactic Management against Kidney Stone

Dhara Patel [α], Ritu Sapra [σ], Grishma Patel [ρ] & Dhananjay Mesharm [ω]

Abstract- Background: Phyto-medicine is regaining interest owing to its advantages over conventional drugs and increasing cases of drug resistance. Moreover, recurrence and persistent side effects of present-day treatment for urolithiasis restrict their use, so an alternate solution using phytotherapy is being sought. The lichen species *Parmelia Perlata (*family *Parmeliaceae)* mentioned in India Materia Medica are useful in treating several ailments, and they are being used in large quantities as a food supplements in India.

Objective: Up to date, the scientific documentation regarding *in-vivo* antiurolithiatic and antimicrobial activity of *Parmelia perlata* has been reported, although it has not reported for *in-vitro* antiurolithiatic as well as an antimicrobial activity up till now. The present study attempted to evaluate phytochemical screening, antimicrobial activity, and anti urolithiatic activity of methanolic lichen extracts of *Parmelia Perlata,* which called "Chandila" by *in vitro* model.

Material and Methods: In this study, the crude extracts were obtained from the *Parmelia perlata* by cold extraction method using methanol as a solvent. The phytochemical tests were being carried out on the extract of lichen. The antimicrobial efficacy was being investigated against various pathogenic bacterial and fungal strains. *In vitro* antiurolithiatic models were investigated through nucleation assay and aggregation assays by spectrophotometric technique. Cystone was being used as a standard drug in the *in vitro* model for anti urolithiatic activity.
Result: The result of the phytochemical tests showed the presence of several biologically active phytochemicals with the highest quantity of alkaloids, flavonoids, and phenols in methanolic extract. The methanolic extract had the highest activity against *P.vulgaris* and *C. tropicalis* at 160 mcg/mL concentration. The maximum percentage of dissolution of existing calcium oxalate crystal was found to be 35% at 40 mg/ml concentration.

Conclusion: Methanolic extract of *Parmelia Perlata* exhibited significant *in vitro* antimicrobial and anti-urolithiatic activity.

Keywords: parmelia perlata, lichen, phytochemicals, antimicrobial, anti-urolithiatic.

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I. Introduction

Urolithiasis, the formation of kidney stone presence of one or more calculi in any location within the urinary tract, is one of the oldest and wide spread diseases known to man.[1] It is a serious, debilitating problem in all societies throughout the world, affecting approximately 12% of the population and, men are three times more prone than women.[2] It is more prevalent between the ages of 20 and 40 in both sexes.[3] Etiology is multifactorial and is strongly related to dietary lifestyle habits or practices.[4] Increased rates of hypertension and obesity also contribute to an increase in stone formation.[5] Kidney stones are hard, solid particles that form in the urinary tract. In many cases, the stones are very small and can pass out of the body without any problems. However, if a stone (even a small one) blocks the flow of urine, excruciating pain may result, and prompt medical treatment may be needed. Recurrent stone formation is a common part of the medical care of patients with stone disease. Calcium-containing stones, especially calcium oxalate monohydrate, calcium oxalate dihydrate and, basic calcium phosphate are the most commonly occurring ones to the extent of 75-90% followed by magnesium ammonium phosphate (Struvite) to the extent of 10-15%, uric acid 3-10% and cystine 0.5-1% [6]. The present-day medical management of nephrolithiasis is either costly or not without side-effects. Invasive procedures for the treatment of nephrolithiasis may cause serious complications and also impose a burden of costs on the healthcare system [7].

Lichens are being used since ancient times as one of the natural drugs [8]. Lichens represent a symbiotic association of a fungus with an algal partner and are important constituents of the ecosystem. Lichens produce characteristic secondary metabolites such as aliphatic, aromatic, and terpenic components which have considerable biological activities such as antiviral, antibacterial, antifungal, antitumor, antioxidant, etc. [9, 10]. *Parmelia perlata* is a well-known lichen of family *Parmeliaceae*. A lichen is an association of an alga and fungus living together in a symbiotic relationship.

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Parmelia perlata is commonly called a Stone flower or Chadila. In India it is mainly found in Himachal Pradesh and West Bengal. It is used as food, fodder and medicine. It is a good pain reliever and is being used as a remedy for the early healing of wounds. It cures many skin diseases and is considered to be an expectorant, astringent, resolvent, laxative, carminative and, aphrodisiac. It is also used in the treatment of fever, cough, dysentery and, renal calculi. This lichen exhibits antimicrobial [11-12], antiviral [13], antitumor [14], antispasmodic [15], antioxidant [16] and antipyretic [17] activities. Its hepatoprotective action[18] has also been reported. Phytochemical studies of *Parmelia perlata* have led to the isolation of various chemical constituents such as atranorin, chloroatranarin, salazinic acid [19], lecanoric acid [20], imbricaric acid [21], lecanora. The study has been undertaken to evaluate *Parmelia perlata* different lichen extracts and cystone as a standard for their possible potential to dissolve experimental kidney stone using a modified in vitro model [22-25] to isolate the chemical constituent responsible for the activity.

II. Material and Methods

a) Source of Plant Materials

The plant material Parmelia perlata (lichen) was collected from the hills of Himachal Pradesh (India). The collected lichen species were identified as *Parmelia perlata* (Rajnegi and Gadgil, 1996) (Figure 1).

b) Source of microorganisms

The organisms studied, *Escherichia Coli* (MTCC No.40), *Pseudomonas aeroginosa* (MTCC No.424), *Staphylococcus aureus* (MTCC No.87), *Proteus vulgaris* (MTCC No.742), *Streptococcus mutans* (MTCC No.497), *Bacillus subtilus* (MTCC No.441), *Staphylococcus epidermidis* (MTCC No.9041), *Micrococcus luteus* (MTCC No.106), Saccharomyces cerevisiae (MTCC No.170), *Candida albicans* (MTCC No.183) and *Candida tropicalis* (MTCC No.1000). The organisms were obtained from MTCC Chandigarh and maintain according to specifications. Sub culturing was done at an interval of 15 days.

c) Extraction of lichen

The collected lichen materials were brought to the laboratory, air-dried for three days, cleaned free of any other plant materials or mosses, and then washed under running tap water. They were oven-dried at 40°C for 42 h and grounded into powder by using a mixer. The powdered samples were stored in sterilized specimen bottles until when needed. Lichen constituents were extracted by cold extraction.

d) Cold extraction

10 g of lichen powder was added to 200 ml of acetone. The mixture was timed thoroughly by using a shaker water bath for five hours, then left at room temperature overnight and filtered using Whatman No. 1 filter paper. The filtrate was collected, and the solvent was removed using rotary evaporator and about 200 mg residues were recovered. The lichen powder that remained on the filter paper was dried and again extracted using 200 ml methanol. From this solvent, about 162 mg of residue was recovered [26].

e) Phytochemical Analysis [27]

Phytochemical analysis for the qualitative detection of alkaloids, glycosides, reducing sugar flavonoids, tannins, and saponins were performed with the extracts.

f) Determination of Antimicrobial Activity

The antimicrobial activity of the lichen extracts was determined using the agar well diffusion method[28] by following the known procedure. Briefly, Nutrient agar was inoculated with the given microorganisms by spreading the bacterial, and fungal inoculums on the media. Wells were made in the agar using the stainless steel borer of 8 mm and filled with 80, 120 and 160 μl of plant extracts. Control wells containing neat solvents (negative control) were also run parallel on the same plate. The plates were incubated at 37°C for 72 hours, and the antimicrobial activity was assessed by measuring the diameter of the zone of inhibition.

g) Evaluation for Anti-Urolithiatic Activity

Behind this activity, the idea was to know the role of plant extract in dissolving the already formed stones nucleus in the renal system. For this artificial calcium oxalate crystals were prepared in the laboratory [29] and semi-permeable membrane was prepared from the egg using standard methods [30-32].

Step-1: Preparation of experimental kidney stones (Calcium oxalate stones) by homogenous precipitation

1.47gm of calcium chloride dihydrate was dissolved in 100 ml distilled water, and 1.34 gm of sodium oxalate was dissolved in 100 ml of 2N H_2SO_4. Both solutions were mixed equally in a beaker to precipitate out calcium oxalate with stirring. An equimolar solution of calcium chloride dehydrate (AR) in distilled water and Disodium hydrogen phosphate (AR) in 10 ml of (2N H_2SO_4) was allowed to react in a sufficient quantity of distilled water in a beaker. The resulting precipitate was calcium phosphate. Both precipitates freed from traces of H_2SO_4 by ammonia solution. Washed the precipitates with distilled water and dried at 60 ^{0}C for 4 hours.

Step- 2: Preparation of semi-permeable membrane from farm eggs

The semi-permeable membrane of eggs lies in between the outer calcified shell and the inner contents like albumin & yolk. The apex of eggs was punctured by a glass rod to squeeze out the entire content. Empty eggs were washed thoroughly with distilled water and placed in a beaker consisting of 2 M HCl for an

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overnight, which caused complete decalcification. Further, washed with distilled water, placed in ammonia solution for neutralization of acid traces in the moistened condition for a while & finally rinsed with distilled water (Figure 2) and stored in the refrigerator at a pH of 7-7.4.

Step-3: Estimation of Calcium oxalate by Spectrophotometrically:

Group I: 1ml of calcium oxalate (1mg/ml) + 1ml of distilled water

Group II: 1ml of calcium oxalate (1mg/ml) + 1ml of Cystone solution

Group III: 1ml of calcium oxalate (1mg/ml) + 1ml of the cold extract of *Parmelia perlata*

All groups were packed together in egg semi-permeable membrane tied with thread at one end and were suspended in a conical flask containing 150 ml, 0.1 M Tris buffer each. At another end of thread tied by a stick placed on the mouth of the conical flask and covered with aluminum foil. All groups were kept in an incubator, pre heated to 37°C for 4 hours, kept for three days. The entire content of each group was removed from the sutured semi permeable membrane and was transferred into the test tube individually. 4ml of 1N H_2SO_4 and 60-80µl of 0.02M $KMnO_4$ were added and kept aside for 2 hours. Color change from dark pink to colorless was observed after 2 hours. The change of color intensity was measured against 620 nm spectrophotometrically. The concentration of undissolved calcium was determined from a standard calibration curve of calcium oxalate by using the measured absorbance readings.

h) Nucleation assay (Turbidity method):

The inhibitory activity of the extracts on the nucleation of calcium oxalate crystals was determined by a spectrophotometric assay. Crystallization was initiated by adding 100 µl of 4 mM calcium chloride and 100 µl of 50 mM sodium oxalate solutions to 0.5 ml of normal human urine, both prepared in a buffer containing 0.5 ml of 0.05 mM Tris buffer and 0.5 ml of 0.15mM NaCl solution at pH 6.5 and 37°C and adjusted to volume by adding 1.5 ml of distilled water. The rate of nucleation was determined by comparing the induction time of crystals (time of appearance of crystals that reached a critical size and thus became optically detectable) in the presence of the extract and that of the control with no extract. The optical density (OD) was recorded at 620nm, and the percentage inhibition calculated as (1-OD (experimental)/OD (control))/100.

i) Aggregation assay

The rate of aggregation of the calcium oxalate crystals was determined by a spectrophotometric assay with slight modifications. The calcium oxalate monohydrate (COM) crystals were prepared by mixing both the solutions of calcium chloride and sodium oxalate of 50 mM each. Both solutions were then equilibrated. The solutions were then cooled to 37°C and then evaporated. The COM crystals were then dissolved with 0.5ml of 0.05mM Tris buffer and 0.5 ml of 0.15mM NaCl solution at pH 6.5 to a final concentration of 1 mg/ml. Absorbance at 620 nm was recorded. The rate of aggregation was estimated by comparing the slope of turbidity in the presence of the extract against control.

III. Results

a) Qualitative Phytochemical Analysis

The present study reveals that lichen extract shows the presence of phytochemical constituents like alkaloids, flavonoids, carbohydrates, glycosides, proteins, saponins, tannins, terpenoids, reducing sugar and volatile oil in solvent extracts as shown in Table 1.

b) Antimicrobial Activity

The potential sensitivity of the extract was obtained against all the microorganisms tested, and the zone of inhibition was recorded. The results obtained were compared against standard antibiotic kanamycin and amphotericin B and presented below in the tabulation drawn (Table 2).

c) Estimation of Calcium oxalate

In kidney stones formation, calcium oxalate and calcium phosphate or other chemicals in the urine form crystals on the inner surfaces of kidneys. This stage is called an initial mineral phase formation. Over the period, crystals may combine to form a small, hard mass called as stones, and the stage is referred to crystal growth. Calcium oxalate stones have been classified into two types, i.e., calcium oxalate monohydrate stones (COM) and calcium oxalate dihydrate stones (COD).

d) Spectrophotometric estimation of calcium oxalate

The extract of *Parmelia perlata* has greater capability to dissolve calcium oxalate as the foremost element for stone forming in urinary tract. Lower percentage indicates more potency in the dissolution of calcium oxalate crystals as shown in Table 3.

e) Nucleation assay

Urine supersaturation attributes to calcium oxalate particles crystallization within the urinary tract. This is a nucleation process where stone-forming salts begins to unite into clusters with the addition of new constituents. Cystone standard solution exhibited stronger inhibition activity than the extract of *Parmelia perlata* in the nucleation of calcium oxalate salts. As *in vitro* crystallization study was performed, since nucleation is an important first step for the initiation of crystals, which then grow and form aggregates, extract of *Parmelia perlata* inhibited crystallization by inhibiting the nucleation of calcium oxalate through disintegrating

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into smaller particles with increasing concentrations of the fraction. The results of nucleation assay confirmed that the extract contained nucleation-preventing agents (Figure 3).

f) Aggregation assay

Calcium oxalate crystals begin to grow, aggregate with other crystals and, retained in the kidney. This is an aggregation process that causes renal injury. The extract of *Parmelia perlata* inhibited formation of COD crystals slightly better compared to Cystone standard solution. COM has a stronger affinity with cell membranes; it may lead to become a higher potential risk for renal calculi formation. This is may be due to the high content of saponins present in *Parmelia perlata*. It has several polyphenolics, e.g., alkaloids, saponins, phenolics, flavonoids, and other phytoconstituents. Saponins are well known to have anti-crystallization properties by disaggregating the suspension of mucoproteins as crystallization promoters. The present investigation will be supportive as additional information to the scientific evidences regarding *in-vitro* studies (Figure 4). Since the mechanism of anti-urolitholytic activity in the extract is exactly unknown to date, the correlation between *in vitro* and *in vivo* studies should be further investigated to reveal the phytochemicals of the extract which are responsible for dissolving or disintegrating renal calculi and for knowing better understanding in the molecular mechanism of lithiolysis.

IV. Conclusion

The present study conclusively demonstrates that *Parmelia perlata* is a good source of various phytochemicals like alkaloids, flavonoids, carbohydrates, glycosides, saponins, tannins, Terpenoids. This study evaluates the antiurolithiatic and antimicrobial activity of methanolic extracts of *Parmelia perlata* Lichen. The work was performed by using an *in vitro* antiurolithiatic model for calculating the percentage dissolution of kidney stones. This study has given primary evidence for *Parmelia perlata* as the lichen which possesses lithotriptic property. From the result Tables, it is also clear that a positive correlation exists between individual extracts and concentration used, in the study. Out of four concentration used we can observe that activity increase as we increase the concentration and, at one point, further no increase in the activity observed. The plant used in the above study also showed good activity when it was compared with the standard drug cystone. The methanolic extract found to be more potent in terms of activity and the authors of the above work recommends the lichen extract for further studies by conducting the *in vivo* model.

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Figure 1: *Parmelia perlata* Lichen



Figure 2: Experimental Egg membrane

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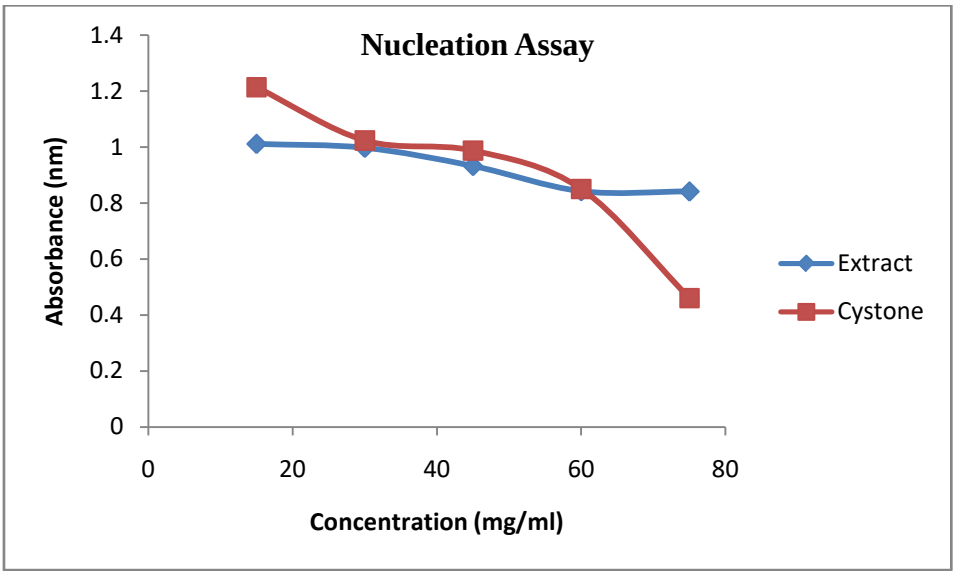


Figure 3: Nucleation assay showed that the various concentration of *Parmelia perlata* lichen extract and Cystone against absorbance at 620nm

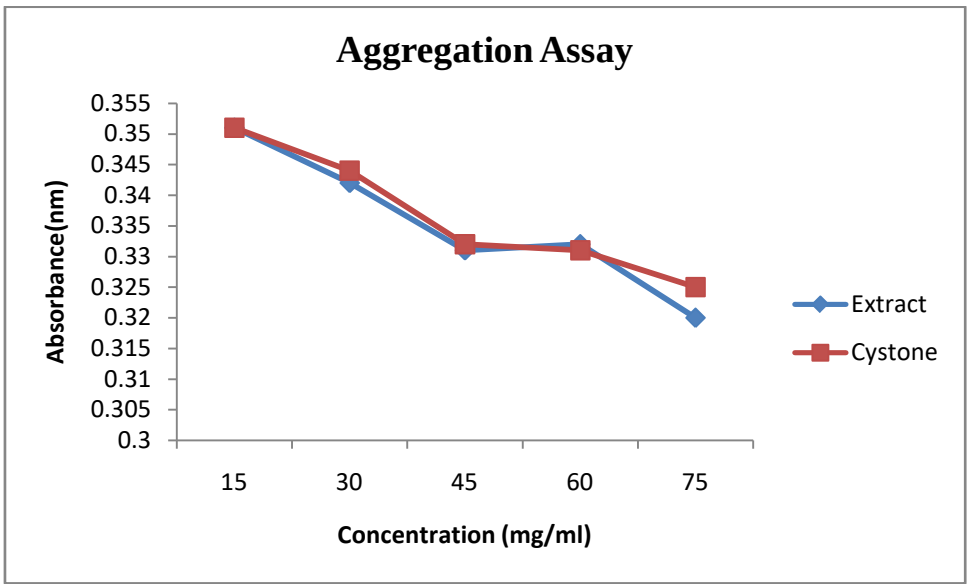


Figure 4: Aggregation assay showed that the various concentration of *Parmelia perlata* extract and Cystone against absorbance at 620nm

Table 1: Phytochemical constituent Present in Extracts

Extract	Alkaloid	Flavanoids	Saponin	Terprnoid	Tannin	Glycoside	Reducing sugar	Volatile oil
Methanolic	+	+	+	+	+	+	+	+

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Table 2: Zone of inhibition of extract and standard

Tested micro organism	Zone of Inhibition ± SD			
	Kanamycin for anti bacterial And Amphotericin B for anti fungal (20 mcg/ml)	80 mcg/ml	120 mcg/ml	160 mcg/ml
E.coli	18 mm ±0.23	10 mm±0.15	13 mm±0.11	15 mm±0.15
S.aureus	21 mm±0.11	13 mm±0.02	14 mm±0.24	18 mm±0.33
P.vulgaris	26 mm±0.16	-	13 mm±0.27	19 mm± 0.31
P.aerouginosa	14 mm±0.32	08 mm± 0.12	11 mm±0.41	15 mm±0.38
B.subtilis	19 mm±0.41	-	-	10 mm±0.21
S.epidermidis	20 mm±0.11	-	10 mm±0.72	16 mm±0.11
M.luteus	15 mm±0.56	-	-	10 mm±0.11
S. cerevisiae	20 mm±0.48	-	15 mm±0.71	17 mm±0.34
C. albicans	17 mm±0.52	-	10 mm±0.19	14 mm±0.36
C. tropicalis	24 mm±0.67	-	15 mm ±0.61	19 mm±0.38

Table 3: Dissolution of calcium oxalate

Groups	Mean ± SD	Weight of calcium oxalate reduced	Dissolution Percentage
Group I	0.130 ± 0.22		
Group II	0.076± 0.32	0.054	42
Group III	0.085± 0.11	0.045	35

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Risk of Arterial and Venous Thromboembolic Events with Bevacizumab, An Antibody Against Vascular Endothelial Growth Factor a (VEGF-A): A Meta-Analysis

By Mihika Ashish Shah, Mandar Kalpesh Shah, Sharan Dharmesh Shah, Harshil Devang Patel, Parshwa Keyur Shah & Dr. Mamta Gupta

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Abstract- *Introduction:* Bevacizumab, a humanized antibody against VEGF, is effective within the treatment of patients with several cancers. However, like several therapeutic agents, important side effects such as arterial thromboembolism, venous thromboembolism, hypertension, neutropenia, proteinuria, and hemorrhage are related to bevacizumab. Thromboembolism is one of the leading causes of morbidity and mortality in patients with cancer. Considerations have arisen relating to the chance of venous and arterial thromboembolism with the novel antiangiogenic agent bevacizumab: a recombinant humanized monoclonal antibody to a vascular endothelial growth factor which is wide employed in cancer treatment.

Keywords: *bevacizumab, avastin, cancer, side effects, arterial thromboembolism, venous thromboembolism.*

GJMR-B Classification: *NLMC Code: WG 610*



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Risk of Arterial and Venous Thromboembolic Events with Bevacizumab, An Antibody Against Vascular Endothelial Growth Factor a (VEGF-A): A Meta-Analysis

Mihika Ashish Shah [α], Mandar Kalpesh Shah [σ], Sharan Dharmesh Shah [ρ], Harshil Devang Patel [ω], Parshwa Keyur Shah [¥] & Dr. Mamta Gupta [§]

Abstract- Introduction: Bevacizumab, a humanized antibody against VEGF, is effective within the treatment of patients with several cancers. However, like several therapeutic agents, important side effects such as arterial thromboembolism, venous thromboembolism, hypertension, neutropenia, proteinuria, and hemorrhage are related to bevacizumab. Thromboembolism is one of the leading causes of morbidity and mortality in patients with cancer. Considerations have arisen relating to the chance of venous and arterial thromboembolism with the novel antiangiogenic agent bevacizumab: a recombinant humanized monoclonal antibody to a vascular endothelial growth factor which is wide employed in cancer treatment.

Methodology: We performed a meta-analysis of published clinical trials of bevacizumab to quantify the risk of Thromboembolic events. Fourteen studies following PRISMA guidelines and matching inclusion and exclusion criteria were collected in which a group of patients was either treated with Bevacizumab and concurrent chemotherapy and another group treated with Placebo and the same chemotherapy. We calculated the Relative risk (RR). $P<0.05$ was considered statistically significant. We used R version 3.3.1 (The R Foundation for Statistical Computing) for the analysis.

Results: Total 12,280 patients were included. Bevacizumab was associated with an increased risk of Arterial Thromboembolic Events at a high dose (R.R=1.6002; 95% C.I: 1.604 to 2.2066) and Venous Thromboembolic Events at high dose (R.R=1.2433; 95% C.I:1.0375 to 1.4448). At the low dose no significant risk was seen.

Keywords: bevacizumab, avastin, cancer, side effects, arterial thromboembolism, venous thromboembolism.

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I. Introduction

Angiogenesis is a process that results in the proliferation of new blood vessels and plays an important role in growth, progression, and metastasis of the tumor. The vascular endothelial growth factor (VEGF) promotes the development of angiogenesis and over expression of the VEGF that is related to poor prognosis in numerous malignancies (1, 2). This process mainly occurs by vascular endothelial growth factor signaling pathway that includes two main target components that are VEGF ligands and VEGF receptors (VEGFRs). Bevacizumab, a humanized monoclonal antibody against VEGF, has shown benefit in the treatment of patients with various malignancies such as metastatic colorectal cancer, non-small-cell lung carcinoma, by many phase III studies. There is much favorable evidence of the benefits of phase II clinical trials in patients with pancreatic cancer, renal cell cancer, and prostatic adenocarcinoma. Though bevacizumab is usually well- tolerated, it may be related to symptomatic side effects like delayed wound healing, hemorrhage, leukoencephalopathy, neutropenia, proteinuria, and nephrotic syndrome, gastrointestinal perforation and congestive heart failure.(3) Bevacizumab conjointly contributes to the event of arterial and venous thromboembolism, a typical complication resulting in morbidity and mortality in patients with malignancy.(4) We hypothesized that sample sizes in randomized control trials were not powered and large to reveal significantly increased risk. Hence we performed a Meta-analysis of published phase 2 and 3 randomized clinical trials of bevacizumab to determine the risk of arterial and venous thromboembolic events.

II. Methodology

a) Data Source

We carried out a systematic search of existing databases and after careful scrutiny by two independent researchers; Fourteen studies were selected for inclusion in the analysis. The search was done based on

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the preferred reporting system for meta-analysis (PRISMA) guidelines.5 An independent review of citations from scientific databases like clinical trials.gov, Pub med central, NCBI, NIH, Cochrane Library, and Google scholar from January 2004 to January 2015 was conducted. Keywords, bevacizumab, Avastin, cancer, human studies, and clinical trial, Arterial Thromboembolic Events (ATE), Venous Thromboembolic Events (VTE) were included in the search. The search was limited only to the articles published in the English language.

b) Data extraction and clinical end points

All study- related Randomized controlled trials (RCTs) using either: A proper method of allocation concealment (e.g., sealed opaque envelopes), Studies that were double-blind, single-blind, studies that were in Phase 2 or Phase 3 trial were only included. Direct comparison of trials with patients treated by Bevacizumab with concurrent chemotherapy and placebo with concurrent chemotherapy in the clinical trials (phase 2 or 3) of cancer were included. The inclusion criteria of the study included the participants greater than or equal to 18 years of age, the studies which included bevacizumab plus a concurrent therapy and placebo with a concurrent therapy, the dose of Bevacizumab should be 2.5mg/kg/week for low dose regimen or greater than or equal to 5mg/kg/week for high dose regimen. The Exclusion criteria of the study included trials including patients treated previously with Bevacizumab or another similar or other malignancies within five years (unless low risk of recurrence), Also the studies with history of abdominal fistula, Gastrointestinal Perforation, intra-abdominal abscess, clinical signs or symptoms of gastrointestinal obstruction, and requirement of parenteral nutrition, non-healing wound, ulcer. Bone fracture, bleeding diathesis, coagulopathy, known CNS disease (except for treated brain metastasis), clinically significant cardiovascular disease, a major surgical procedure within 28 days of enrollment, or anticipated to occur while participating in the study were excluded from the analysis, unpublished research work or trials were excluded. The outcomes were measured for Thromboembolic events, according to National Cancer Institute Common Terminology Criteria Version 3.The outcome was measured after six cycles for six studies and till overall survival in eight studies. Data were extracted from studies meeting the above criteria. Those studies in which data was unclear asked from respective authors. In some studies, data could not obtain by the inquiry were excluded. Authors assured that the study included was only those in which allocation of both the groups were adequately randomized, and there was not any conflict of interest as well as match to inclusion and exclusion criteria. Also, the concurrent treatment was the same for the group with Bevacizumab therapy and Placebo therapy.

c) Statistical analysis

The outcome of the occurrence of Arterial Thromboembolic Event (ATE) and Venous Thromboembolic Event(VTE) was recorded from both the groups (Bevacizumab and Placebo), and Relative Risk (RR) was calculated with 95% Confidence Interval and funnel as well as forest plot was obtained. R version 3.3.1 (The R

Foundation for Statistical Computing) was used for analysis. A P-value less than 0.05 were considered significant. The presence of small-study effects or publication bias was assessed by funnel plot and eggers value was also calculated. P-value of eggers test, >0.05 is considered to have less publication bias.

III. Results

Total 14 randomized clinical trials were included for Meta-analysis.

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Table 1: Characteristics of randomized controlled clinical trials included in the meta-analysis, including Arterial Thromboembolic Events (ATE)

Study Name	Trial Phase	Underlying Malignancy	Bevacizumab Dose	Concurrent Treatment
A. Ohtsu 2011 et al[6]	3	Advanced gastric cancer	2.5mg/kg/every week	Fluropyrimidine-Cisplatib
B.Escudier 2007 et al [7]	3	metastatic renal cell carcinoma	5mg/kg/week	interferon alfa
C.Aghajanian 2012 et al [8]	3	Recurrent Epithelial Ovarian, Primary Peritoneal, or Fallopian tube cancer	5mg/kg every week	gemcitabine plus carboplatin;
D.Miles 2010 et al[9]	3	HER 2- metastatic Breast cancer	2.5mg/kg/week	Docetaxel
D.Miles 2010 et al[9]	3	HER 2- metastatic Breast cancer	5mg/kg/week	Docetaxel
E.Cutsem 2009 et al[10]	3	Metastatic Pancreatic Cancer	2.5mg/kg/week	Gemcitabine and erlotinib
F.Kabbinavar 2005 et al[11]	2	Metastatic Colon Cancer	2.5mg/kg every week	Bolus fluorouracil and leucovorin
H. Hurwitz 2004 et al[12]	2	Metastatic Colon Cancer	2.5mg/kg/week	Irinotecan, bolus fluorouracil and leucovorin
H.Hurwitz 2005 et al[13]	3	Metastatic Colorectal Cancer	2.5mg/kg/week	irinotecan/fluorouracil/leucovorin
H.Hurwitz 2013 et al[14]	3	Metastatic Colorectal Cancer	5mg/kg/week	Chemotherapy
M .Reck 2009 et al[15]	3	Nonsquamous Non–Small-Cell Lung Cancer	2.5mg/kg every week	Cisplatin and gemcitabine
M .Reck 2009 et al[15]	3	Nonsquamous Non–Small-Cell Lung Cancer	5mg/kg every week	Cisplatin and gemcitabine
N. Robert 2011 et al[16]	3	HER 2- locally recurrent or metastatic Breast cancer	5mg/kg every week	Capecitabine taxane anthracycline
R.Burger 2011 et al[17]	3	Ovarian Cancer	5mg/kg every week	Carboplatin Pacitaxel

Table 2: Characteristics of randomized controlled clinical trials included in the meta-analysis, including Venous Thromboembolic Events (VTE)

Study Name	Trial Phase	Underlying Malignancy	Bevacizumab Dose	Concurrent Treatment
A .Ohtsu 2011 et al[6]	3	Advanced gastric cancer	2.5mg/kg/every week	Fluropyrimidine-Cisplatib
B .Escudier 2007 et al[7]	3	metastatic renal cell carcinoma	5mg/kg/week	interferon Alfa
C .Aghajanian 2012 et al[8]	3	Recurrent Epithelial Ovarian, Primary Peritoneal, or Fallopian Tube cancer.	5mg/kg every week	gemcitabine plus carboplatin;
C .Zhou 2015 et al[18]	3	Recurrent Non squamous non small cell lung cancer	5mg/kg every week	Pacitaxel or carboplatin
D .Miles 2010 et al[9]	3	HER 2- metastatic Breast cancer	2.5mg/kg/week	Docetaxel
D .Miles 2010 et al[9]	3	HER 2- metastatic Breast cancer	5mg/kg/week	Docetaxel
E .Cutsem 2009 et al[10]	3	Metastatic Pancreatic Cancer	2.5mg/kg/week	Gemcitabine and erlotinib
F .Kabbinavar et al 2005[11]	2	Metastatic Colon Cancer	2.5mg/kg every week	Bolus fluorouracil and leucovorin
H. Hurwitz 2004 et al[12]	2	Metastatic Colon Cancer	2.5mg/kg/week	Irinotecan, bolus fluorouracil and leucovorin
H .Hurwitz 2005 et al[13]	3	Metastatic Colorectal Cancer	2.5mg/kg/week	irinotecan/fluorouracil/leucovorin
H .Hurwitz 2013 et al[14]	3	Metastatic Colorectal Cancer	5mg/kg/week	Chemotherapy
H .Kindler 2010 et al[19]	3	advanced pancreatic cancer	5 mg/kg/week	Gemcitabine
H .Kindler 2012 et al[20]	2	Malignant Mesothelioma	5mg/kg every week	gemcitabine cisplatin
M .Reck 2009 et al[15]	3	Nonsquamous Non–Small-Cell Lung Cancer	2.5mg/kg every week	Cisplatin and gemcitabine
M .Reck 2009 et al[15]	3	Nonsquamous Non–Small-Cell Lung Cancer	5mg/kg every week	Cisplatin and gemcitabine
N .Robert 2011 et al[16]	3	HER 2- locally recurrent or metastatic Breast cancer	5mg/kg every week	Capecitabine taxane anthracycline
R. Burger 2011 et al[17]	3	Ovarian Cancer	5mg/kg every week	Carboplatin Pacitaxel

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a) *Relative Risk of Arterial Thromboembolic Events (ATE) with Bevacizumab at a low dose (2.5mg/kg/cycle) versus Placebo*

There are seven clinical trials for determining the Risk of Arterial Thromboembolic Events (ATE), including 3691 patients (1866 in the Bevacizumab group and 1825 in the placebo group). The Relative Risk of Arterial Thromboembolic Events (ATE) with patients treated with Bevacizumab and concurrent therapy was 1.0974 times more than placebo and concurrent therapy with 0.856 to 1.4062 C.I and p- value is 0.4625 which is statistically insignificant. P-value of Egger's test is 0.5725.

b) *Relative Risk of Arterial Thromboembolic Events (ATE) with Bevacizumab at a high (5mg/kg/cycle) versus Placebo*

There are seven clinical trials for determining the Risk of Arterial Thromboembolic Events (ATE), including 8457 patients (4575 in the Bevacizumab group and 3882 in the placebo group). The Relative Risk of Arterial Thromboembolic Events (ATE) with patients treated with Bevacizumab and concurrent therapy were 1.6002 times more than placebo and concurrent therapy with 1.1604 to 2.2066 C.I and p- value is 0.0041which is statistically significant. P-value of Egger's test is 0.67535.

c) *Relative Risk of Venous Thromboembolic Events (VTE) with Bevacizumab at a low dose (2.5mg/kg/cycle) versus Placebo*

There are seven clinical trials for determining the Risk of Venous Thromboembolic Events (VTE), including 3691patients (1866 in the Bevacizumab group and 1825 in the placebo group). The Relative Risk of Venous Thromboembolic Events (VTE) with patients treated with Bevacizumab and concurrent therapy was 0.9143 times more than placebo and concurrent therapy with 0.7617 to 1.0975 C.I and p- value is 0.3361 which is statistically insignificant. P-value of Egger's test is 0.457.

d) *Relative Risk of Venous Thromboembolic Events (VTE) with Bevacizumab at a high dose (5mg/kg/cycle and above) versus Placebo*

There are ten clinical trials for determining the Risk of Venous Thromboembolic Events (VTE), including 9379patients (5045 in the Bevacizumab group and 4334 in the placebo group). The Relative Risk of Venous Thromboembolic Events (VTE) with patients treated with Bevacizumab and concurrent therapy was 1.2243 times more than placebo and concurrent therapy with 1.0375 to 1.4448 C.I and p-value is 0.0166 which is statistically significant. P-value of Egger's test is 0.5878.

Assessment of Publication Bias

As indicated by the p-value of Egger's Test and funnel plots, no publication bias was reported in the selection of studies (Supplementary File).

IV. Discussion

Thromboembolic events are one of the major causes of death in patients with cancer. This paper has tried to show the thromboembolic events associated with bevacizumab- : Anti VEGF at both high and low doses. The safety of this drug is still not clear due to lack of powered clinical trials. So to overcome this we have performed meta-analysis, which includes 14 randomized clinical trials including, 12,280 patients. However, many previous systematic reviews and meta-analysis showed the adverse effect of bevacizumab but not as per the dosage. In this paper, we attempted to associate thromboembolic events with bevacizumab, at different doses by using meta-analysis.

Due to the anti-VEGF effect of bevacizumab it may result in the development of venous thromboembolism. Bevacizumab may expose subendothelial procoagulant phospholipids resulting in thrombosis by inhibiting VEGF induced endothelial regeneration and may reduce the production of nitric oxide and prostacyclin and also causes inhibition of VEGF that causes overproduction of erythropoietin that leads to increased hematocrit and blood viscosity . (21-23) Also bevacizumab could increase the discharge of procoagulant from the neoplasm into the blood due to its cytotoxic effect and also increase the expression of pro-inflammatory cytokines leading to damage and in situ thrombus formation. (24) The hallmark behind any arterial thromboembolism is that the instability of atherosclerotic plaque, activation of platelets, and decreased anti- inflammatory effect of VEGF exposure leading to plaque instability and ruptures, which leads to thromboembolism. (24, 25) Our meta-analysis shows that high dose bevacizumab is related to a significant increased risk of arterial occlusion in patients who received treatment for metastatic cancers of lung, ovarian, colorectal, and pancreatic and kidney that was similar to the study of Scappaticci, Frank A., Jamey R. Skillings, Scott N. Holden, Hans-Peter Gerber, Kathy Miller, Fairooz Kabbinavar, Emily Bergsland. Our meta-analysis additionally shows the increased risk of venous occlusion with a high dose of bevacizumab that was similar to the study of Shobha rani Nalluri, David Chu, Roger Keresztes, Xiaolei Zhu, Shenhong Wu. Due to the increasing use of angiogenesis inhibitors in patients with many metastatic cancers owing to the associated survival benefit, it's important that oncologists monitor and manage these side effects befittingly to confirm that patients receive maximum benefit from bevacizumab therapy.

V. Conclusion

The association of Thromboembolic events with new agents presents a challenge for recognition as a result of several RCTs might not be powered to reveal a significant relationship. Our meta-analysis has overcome

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this limitation of individual trials and incontestable that bevacizumab is also related to a considerably increased risk of arterial and venous Thromboembolic events at the high dose. This finding can facilitate physicians and patients to acknowledge the danger of venous thromboembolism with the administration of bevacizumab at high doses, and so thromboembolic events ought to be monitored.

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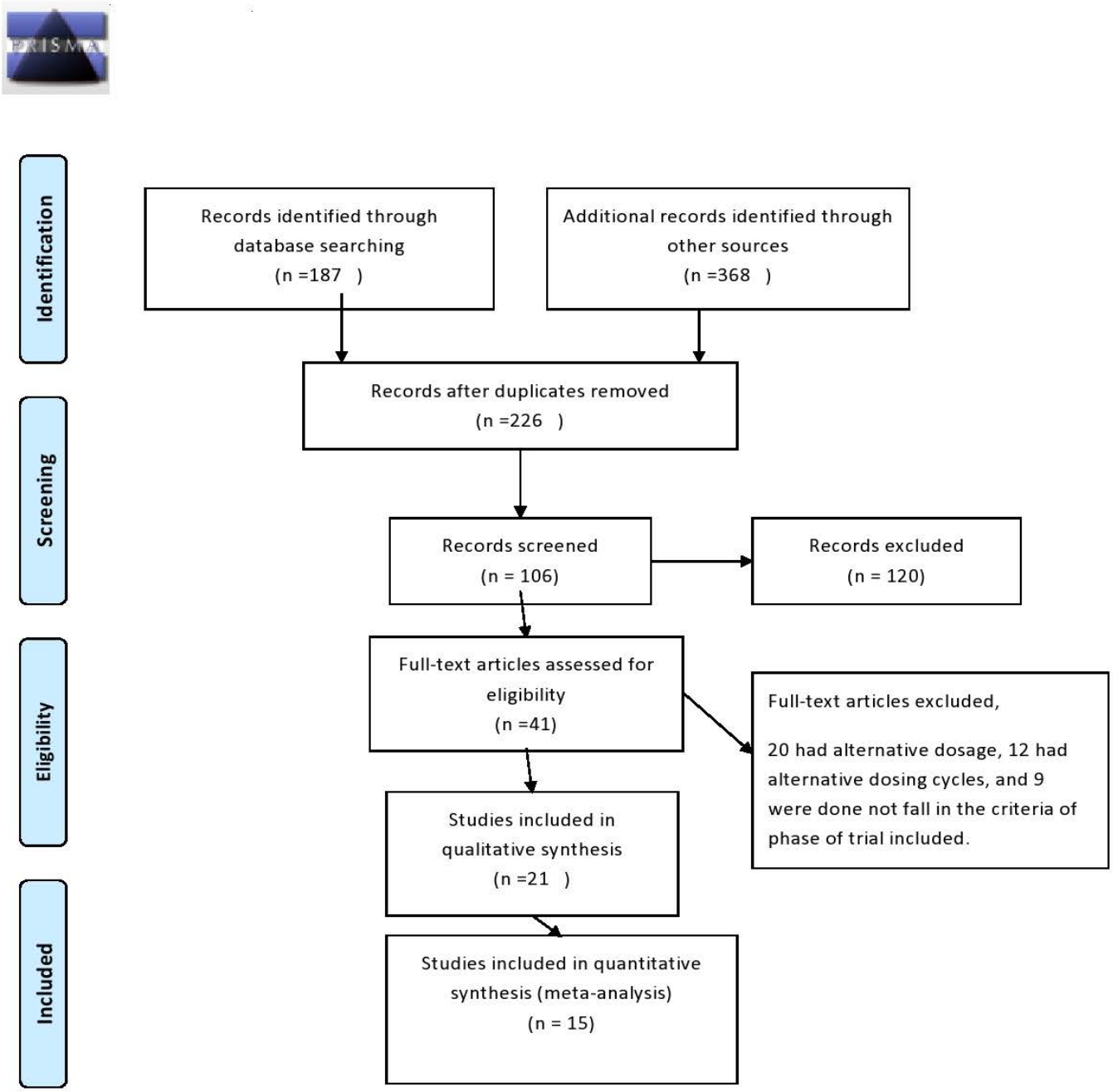


Figure 1: PRISMA flow diagram of included articles

Prisma 2009 Flow Digram

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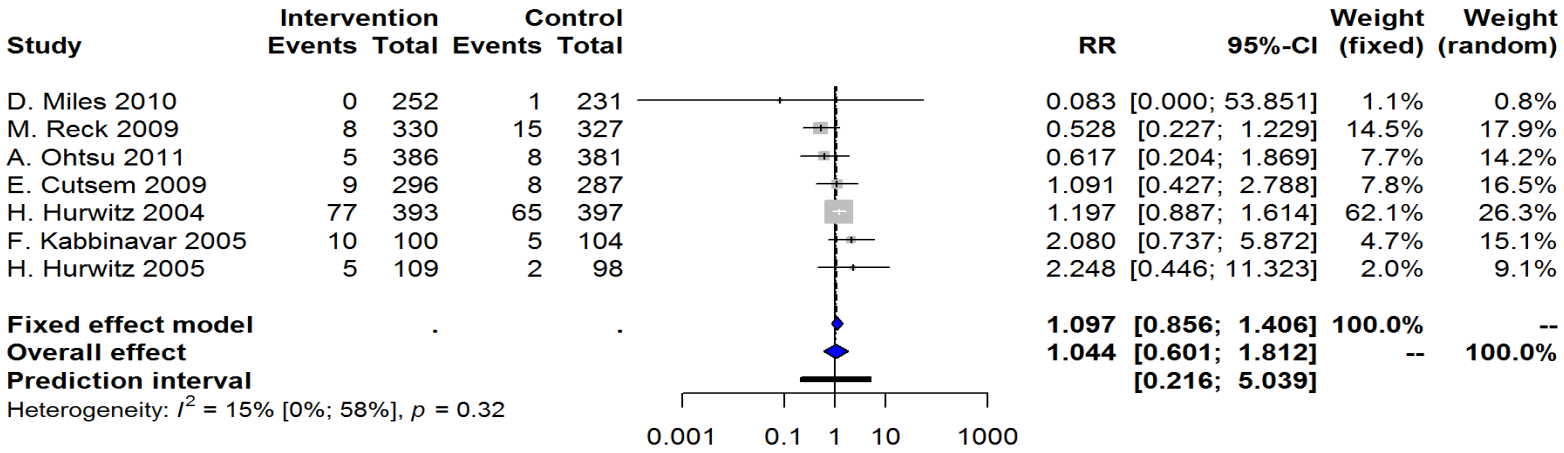


Figure 2: Relative Risk of Arterial Thromboembolic Events (ATE) with Bevacizumab at low dose (2.5mg/kg/cycle) versus Placebo

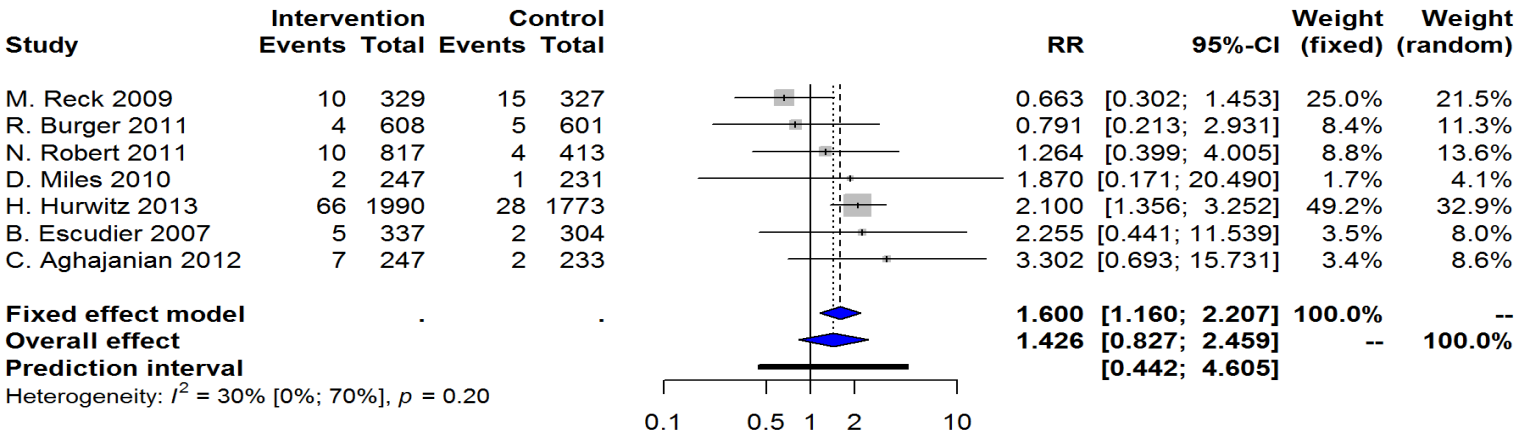


Figure 3: Relative Risk of Arterial Thromboembolic Events (ATE) with Bevacizumab at high (5mg/kg/cycle) versus Placebo

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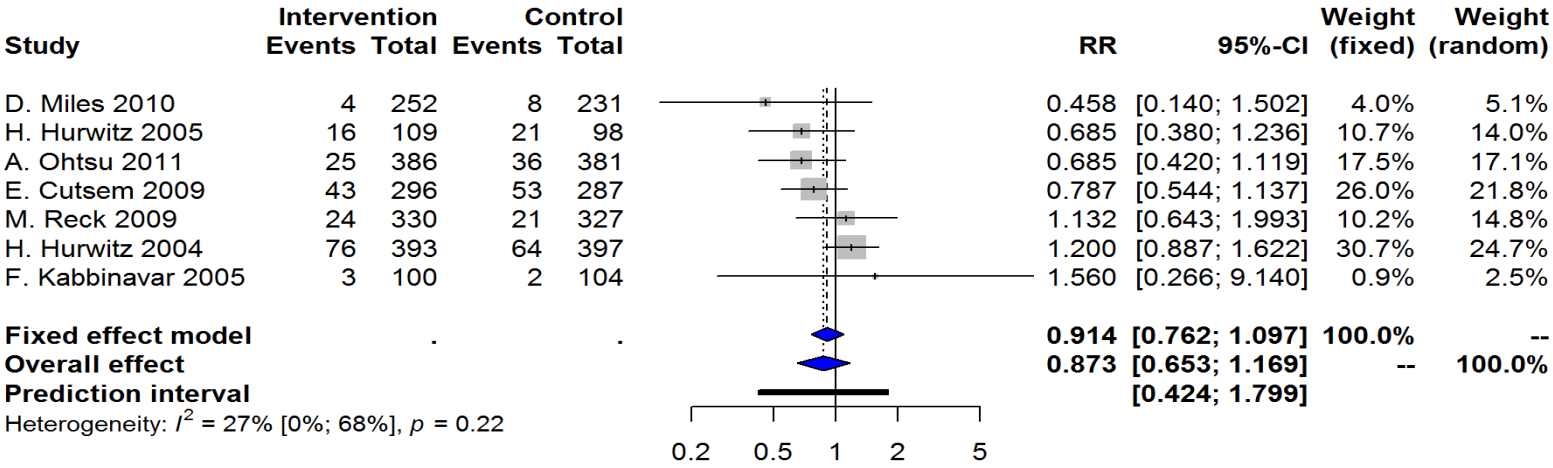


Figure 4: Relative Risk of Venous Thromboembolic Events (VTE) with Bevacizumab at low dose (2.5mg/kg/cycle) versus Placebo

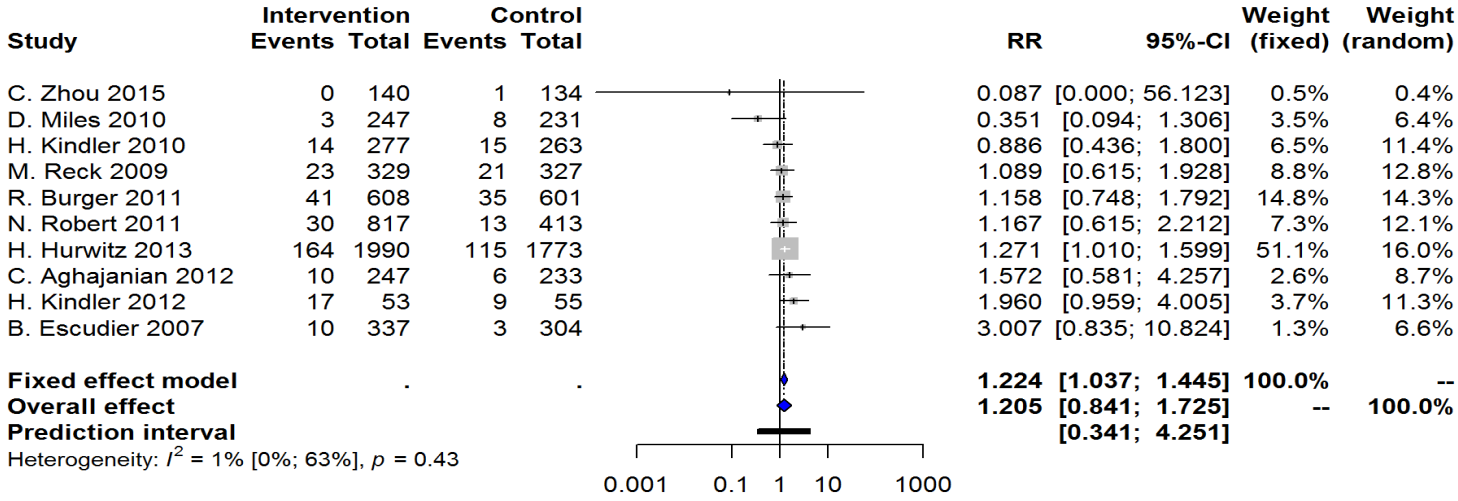
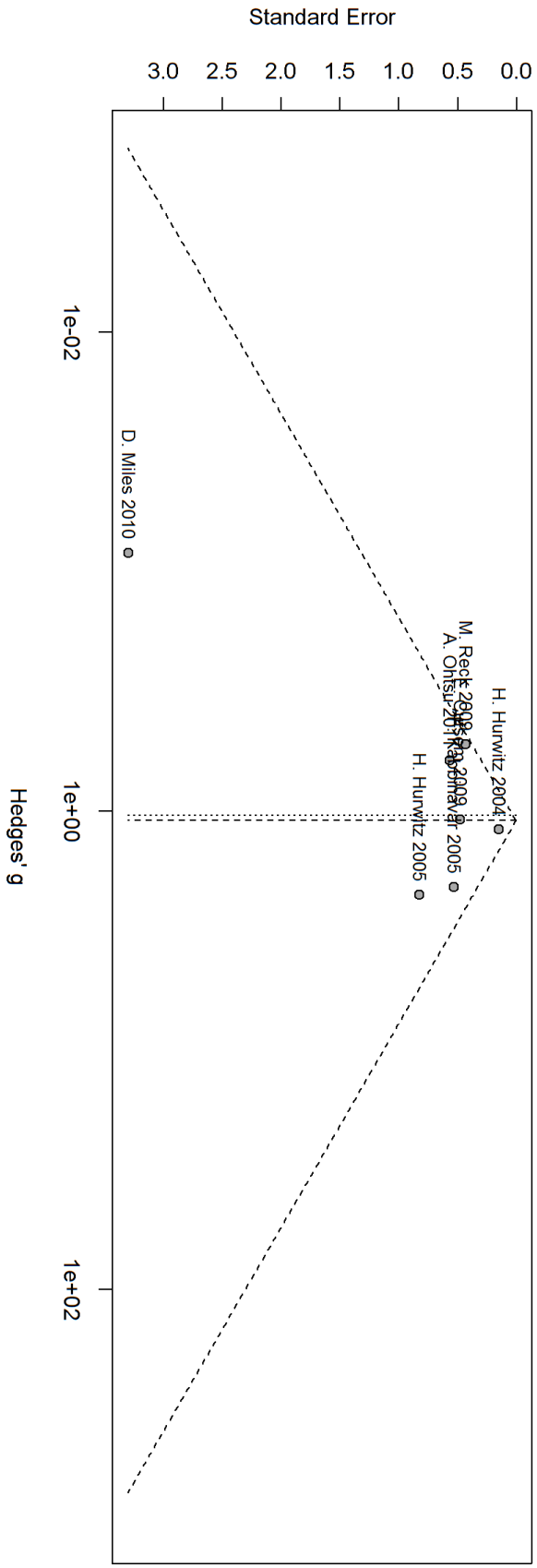


Figure 5: Relative Risk of Venous Thromboembolic Events (VTE) with Bevacizumab at high dose (5mg/kg/cycle and above) versus Placebo

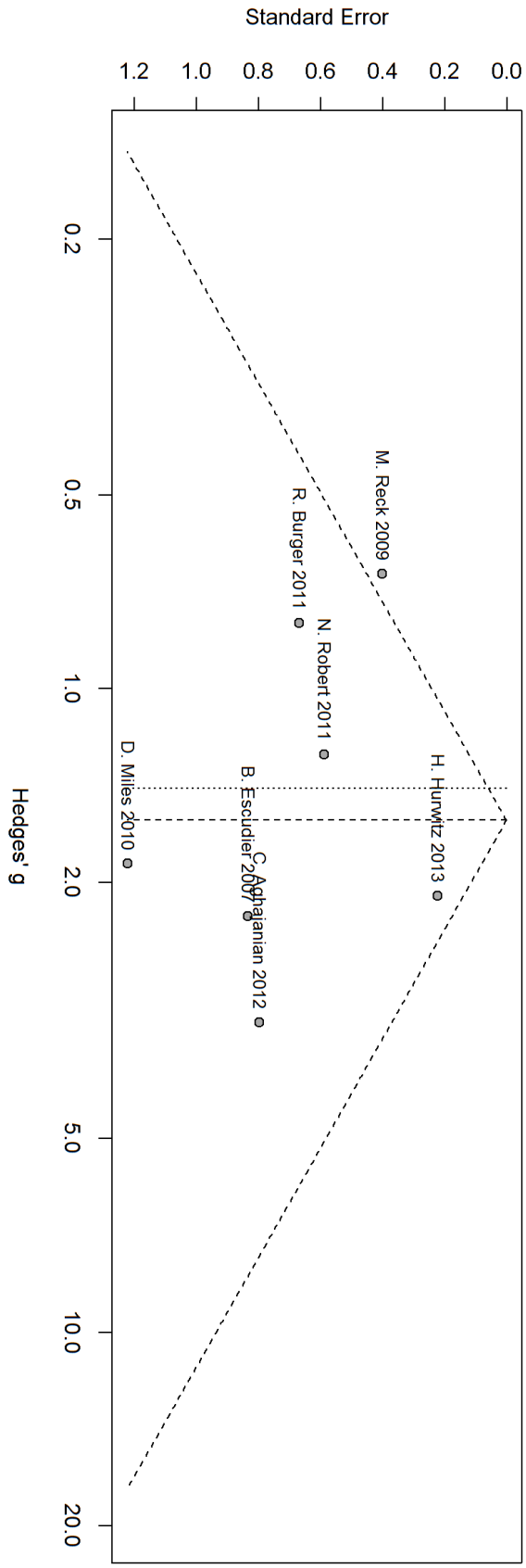
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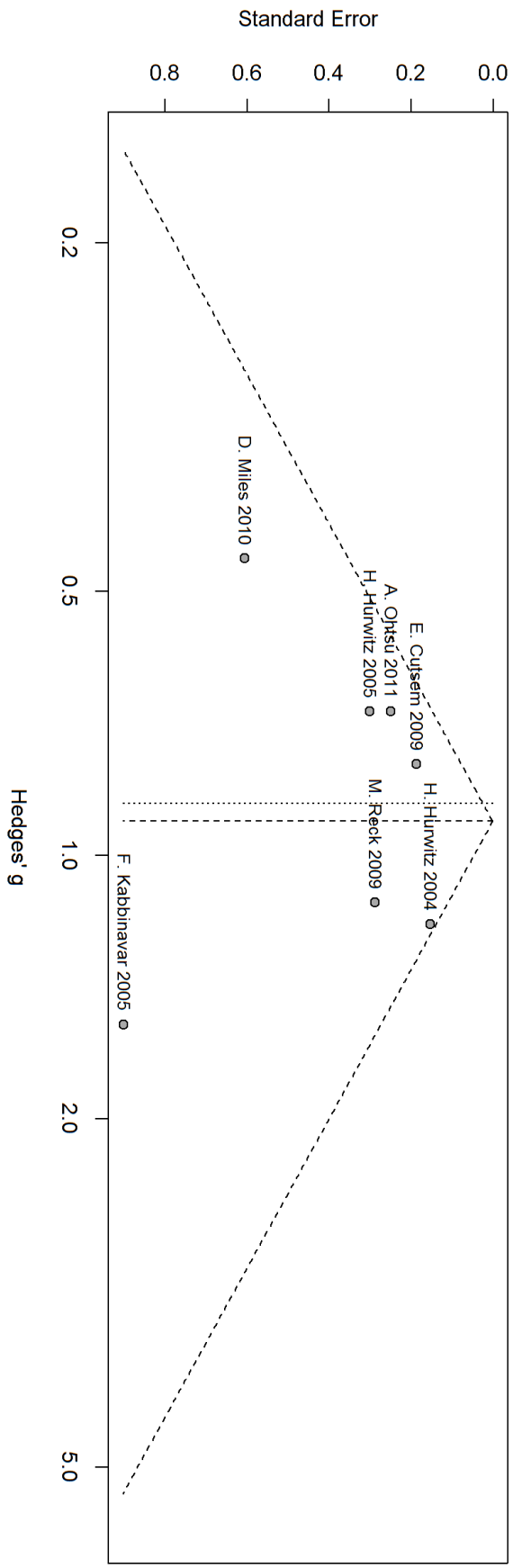
Funnel Plot of Arterial Thromboembolic Events at Low Dose

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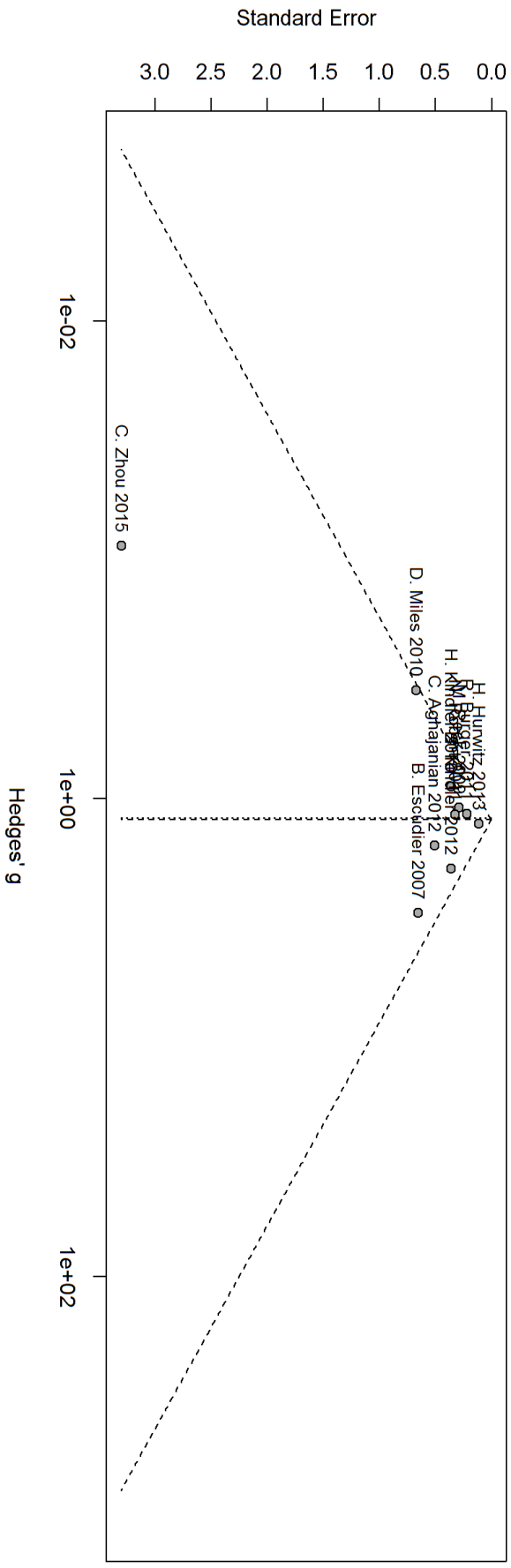
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14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.



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Mistakes to avoid:

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.



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Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

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Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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