EVALUATION OF IN *VIVO* AND IN *VITRO* ANTI-INFLAMMATORY ACTIVITY OF NOVEL ISOXAZOLE SERIES

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Abstract.
Many Isoxazole derivatives are stated to have good anti-inflammatory activity. Chalcones are prepared by the reaction of aromatic aldehydes with aromatic ketones in aqueous alcoholic alkaline medium. Then these are made to react with hydroxylamine hydrochloride and sodium acetate to prepare isoxazole derivatives. The prepared isoxazole compounds are subjected to inflammatory activity by in-vitro and in-vivo methods. All compounds exhibited anti-inflammatory activity among tested 25 isoxazole derivatives. out of these 25 isoxazole derivatives 7 of them shows significant anti inflammatory activity.

Key words – isoxazole, anti-inflammatory, plethysmography, Chalcones, Carrageenan

1. Introduction

The isoxazoles are strong bases and able to exist in both charged (protonated) and uncharged (unprotonated) forms[1]. Many Isoxazole derivatives are stated to have good anti-inflammatory activity. Amgad G. Habeeb et al.[2] also reported a isoxazolines belonging various substitutes at 3rd position of the aromatic ring, which made for analysis as analgesic, anti-inflammatory agents. In this light hereby prepared many Isoxazole derivatives which screened for anti-inflammatory activity by *in-vitro* and *in vivo* methods.

Inflammation is a protective response that involves immune cells, blood vessels, and molecular mediators. The word 'inflammation' comes from the Latin word *inflammare.* The purpose of inflammation is to eliminate the initial cause of cell injury, clear out necrotic cells and tissues damaged from the original insult and the inflammatory process, and to initiate tissue repair.[5] Inflammatory abnormalities are a large group of disorders that underlie a vast variety of human diseases. The immune system is often involved with inflammatory disorders, demonstrated in both allergic reactions and some myopathies, with many immune system disorders resulting in abnormal inflammation. Non-immune diseases with etiological origins in inflammatory processes include cancer, atherosclerosis, and ischaemic heart disease. A large variety of proteins are involved in inflammation, and any one of them is open to a genetic mutation which impairs or otherwise deregulates the normal function and expression of that protein.

Examples of disorders associated with inflammation include Acne vulgaris, Asthma, Celiac disease, Autoimmune diseases, Auto inflammatory diseases, Chronic prostatitis, Vasculitis, Glomerulonephritis, Hypersensitivities, Inflammatory bowel diseases, Pelvic inflammatory disease Reperfusion injury, Rheumatoid arthritis, Sarcoidosis, Transplant rejection, Interstitial cystitis.

Among the many methods used for screening of anti-inflammatory drugs, one of the most commonly employed techniques is based upon the ability of such agents to inhibit the edema produced in the hind paw of the rat after injection of a phlogistic agent. Many phlogistic agents (irritants) have been used, such as brewer's yeast, formaldehyde, dextran, egg albumin, kaolin, sulfated polysaccharides like carrageenan or naphthoylheparamine. The effect can be measured in several ways. The hind limb can be dissected at the talocrural joint and weighed. Usually, the volume of the injected paw is measured before and after application of the irritant and the paw volume of the treated animals is compared to the controls.

Various devices have been developed for plethysmography of the paw. Winter et al. (1963) used mercury for immersion of the paw. A more sophisticated apparatus has been described by Hofrichter et al (1969). Alpermann and Magerkurth (1972) described an apparatus based on the principle of transforming the volume being increased by immersion of the paw into a proportional voltage using a pressure transducer. Webb and Griswold (1984) reported a sensitive method of measuring mouse paw volume by interfacing a Mettler DeltaRange top-loading balance with a microcomputer.

This model is based on the principle of release of various inflammatory mediators by carrageenan.[5] Edema formation due to carrageenan in the rat paw is biphasic, where in the initial phase the release of histamine and serotonin takes place. The second phase is due to the release of prostaglandins, protease and lysosome .Subcutaneous injection of carrageenan into the rat paw produces inflammation resulting from plasma extravasation, increased tissue water and plasma protein exudation, along with neutrophil extravasation, due to the metabolism of arachidonic acid. The first phase begins immediately after injection of carrageenan and diminishes in two hours, while the second phase begins at the end of first phase and remains through three to five hours.

Xylene-induced mice ear edema reflects the oedematization occurred during the early stages of acute inflammation, which is probably related with the release of inflammation mediators. Severe vasodilation, edema changes of skin and infiltration of inflammatory cells are detected as signs of acute inflammation after topical application of xylene. In xylene-induced ear edema model, the application of xylene induces neurogenous edema, which is partially associated with the substance P, an decapeptide of central and peripheral nervous system, and acts as a neurotransmitter or neuromodulator in several physiological processes. Substance P is released from the neurons in the midbrain in response to stress, where it facilitates dopaminergic neurotransmission from sensory neurons in the spinal cord against noxious stimuli and excites dorsal neurons. In the periphery, release of substance P from sensory neurons causes vasodilatation and

plasma extravasations suggesting its role in neurogenous inflammation. Thus, it can cause the swelling of ear in the mice**.**

In vitro anti inflammatory activity was measured by protein denatutration method using fresh egg albumin. In their natural state, proteins like egg albumin and milk casein are soluble in water. There are several mechanisms that destroy these properties. Heat, acids, strong alkalis, alcohol, urea, salicylate, and ultraviolet light are among the more common ways that proteins become denatured. A denatured protein unfolds, as many of the hydrogen bonds that preserve the three dimensional structure of the protein are broken. Instead of a uniform solution of molecules that are all the same shape, in a denatured protein, the molecules can take a staggering number of different shapes (on the order of 10^{20} different shapes, depending on the size of the protein molecule). It has been reported that one of the features of several non-steroidal, anti-inflammatory drugs, is their ability to stabilize (prevent denaturation) heat-treated albumin at the physiological pH (pH: 6.2 – 6.5). Valdecoxib, parecoxib, cycloserine etc are available drug with isoxazole nucleus having anti-inflammatory properties.In this light hereby prepared many isoxazole derivatives which screened for anti-inflammatory activity by in-vitro and in vivo studies.

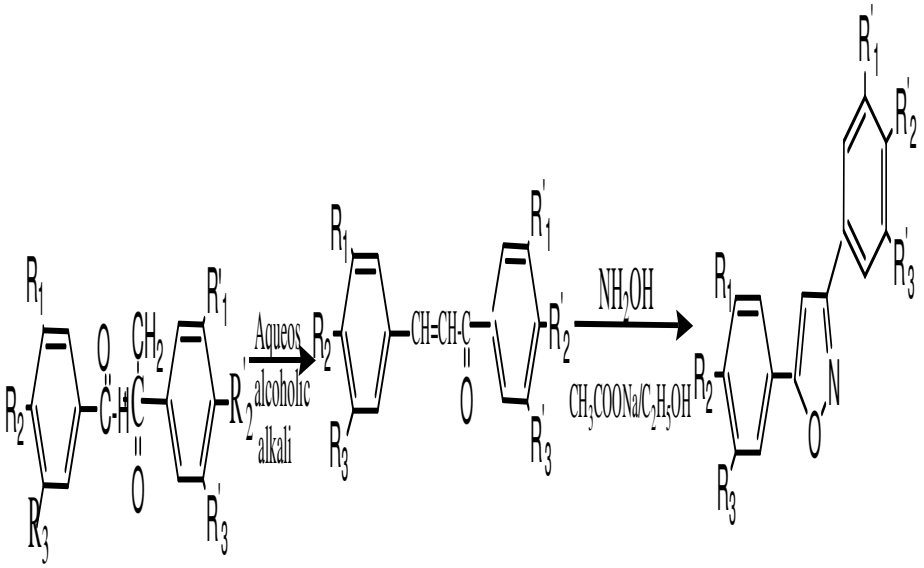
2. Materials and methods

2.1 Chemicals used

All chemicals used for this project were of AR grade and LR grade.

2.2 Methodology for Synthesis: [7, 8, 9]

Chalcones are prepared by the reaction of aromatic aldehydes with aromatic ketones in aqueous alcoholic alkaline medium. Then these are made to react with hydroxylamine hydrochloride and sodium acetate to prepare title compounds. The prepared isoxazole compounds are subjected to inflammatory activity by *in-vitro* and *in vivo* methods.



2.3 Pharmacological screening

2.3.1 Animals

Young Swiss-Albino mice aged about 4–5 weeks with average weight of 25–35 gm and adult wistar albino Rats of either sex having average weight of 150-250 gm. were used for the experiment and maintained in the animal house of the Pushpagiri College of pharmacy. They were housed in standard cages under standard environmental conditions of room temperature at 24 ± 1°C and 55-65% relative humidity with 12 hour dark light cycle and provided with standard food for rodents and water *ad libitum*. The experimental protocol was approved by the institutional animal Ethics committee in Pushpagiri College of pharmacy and was performed according to the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) Guidelines.

2.3.2. Methodology for In vivo anti-inflammatory activity[4]

2.3.2.1 Xylene-Induced Ear Edema Thickness.

The animals (mice) can be divided into five groups (n = 6), fasted overnight and allowed free access to water. The animals are administered with drugs to respective groups. One hour later, each animal received 30µL of xylene using micropipette on anterior and posterior surfaces of the right ear. The left ear is considered as control. Diclofenac (15 mg/Kg) used as standard. Again after one hour later, the thickness of the ear is determined using Digimatic Caliper. The percentage of ear edema is calculated based on the left ear without xylene.

2.3.2.2 Carrageenan Induced Paw edema[11]

Wistar Albino rats weighing 150 -250 g were used for animal studies. Acute inflammation is provided by injection of 0.1ml of 1% carrageenan into the sub plantar surface of rat hind paw. The animals were grouped into Group 1 served as control 1%sodium CMC, Group 2 received standard diclofenac and 0.1ml of carrageenan, Group 4 received test group and 0.1ml of carrageenan. The paw volume was measured at 0^{th} and 4^{th} hours.

2.3.3 Methodology for In vitro anti-inflammatory activity[12]

The reaction mixture consisted of 0.2 ml of egg albumin (from fresh hen's egg), 2.8 ml of phosphate buffered saline (pH 6.4) and 2 ml of varying concentrations of the test series, by which the concentrations (100µg/ml) . Similar volume of double-distilled water served as control. Then the mixtures were incubated at 37°C ± 2°C in a biological oxygen demand incubator for 15 min and then heated at 70°C for 5 min. After cooling, their absorbance was measured at 660 nm by using vehicle as blank. Diclofenac sodium (100µg/ml) used as reference. The percentage inhibition of protein denaturation was calculated by using the following formula:

% inhibitionof protein denaturation = 100 × ([Vt/Vc] −1). Where, Vt = absorbance of test sample, Vc = absorbance of control.

2.3.4 Statistical analysis

All the values are expressed in mean ± SEM. Statistical significance was calculated by one way ANOVA with Dennett's test.

3. RESULTS:

Figure: 1 anti-inflammatory activity of isoxazole derivatives on xylene induced ear edema model

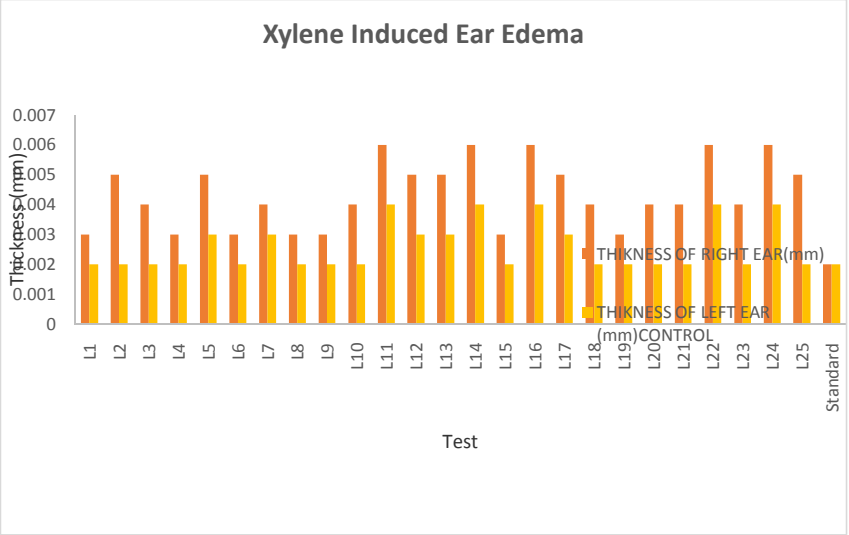


Figure: 2 anti-inflammatory activity of isoxazole derivatives on carrageenan induced paw edema model

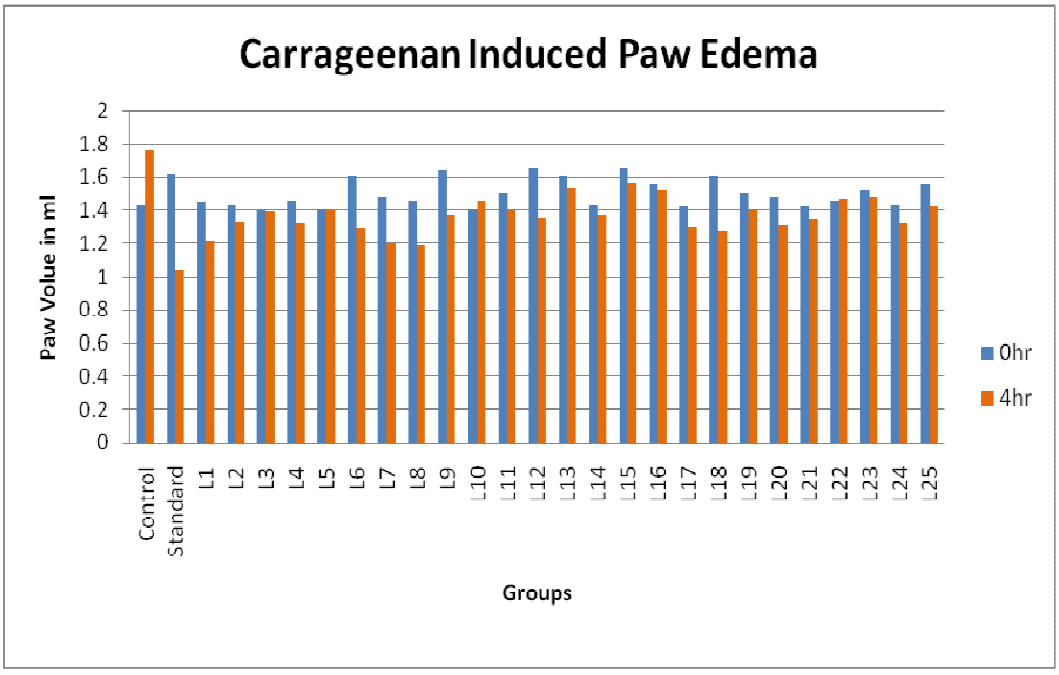
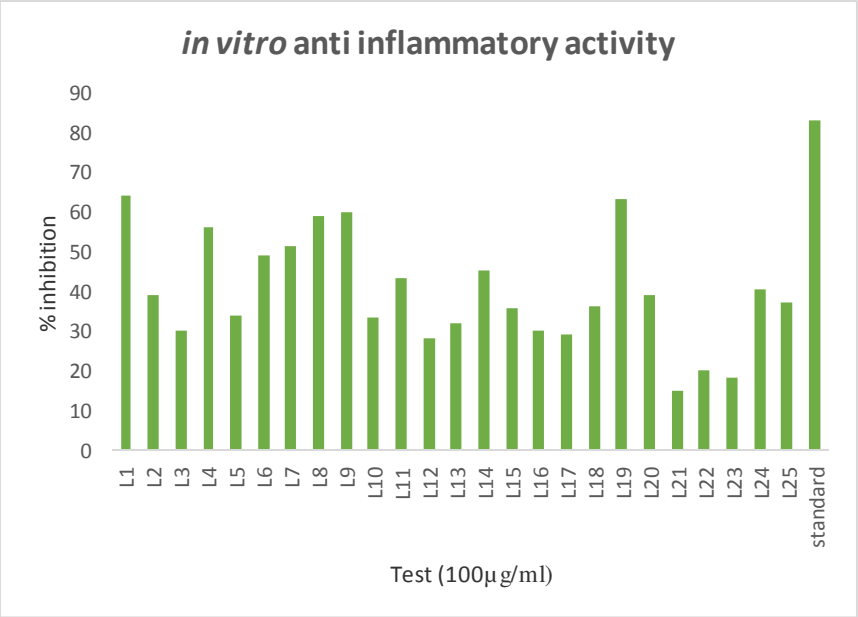
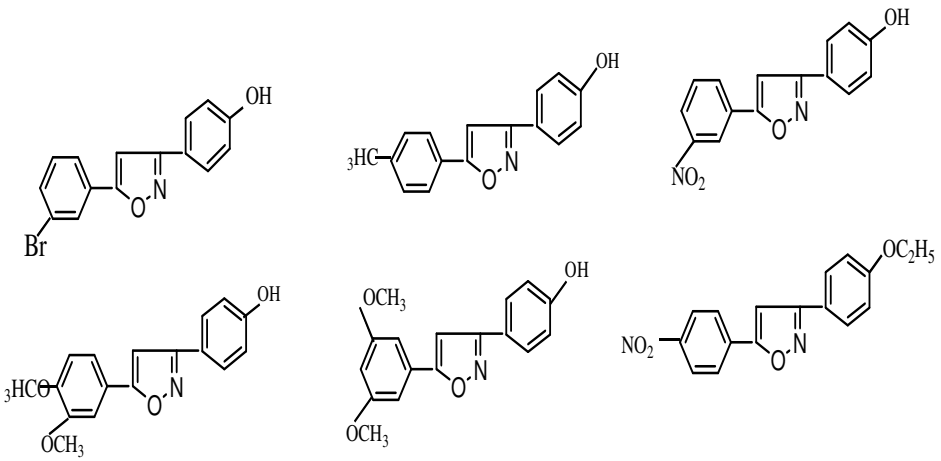


Figure: 3 *in-vitro* anti-inflammatory activity of isoxazole derivatives by inhibition of heat induced protein denaturation method



None of the tested compounds are superior to Diclofenac sodium which was the standard. All compounds exhibited anti-inflammatory activity among tested 25 isoxazole derivatives have polar functional group at para position of phenyl ring at 3-C of isoxazole ring. In addition to this when bromine was in meta position of phenyl ring at 5-C of isoxazole exerted anti-inflammatory activity ,while changing the position of bromine from meta to para position in phenyl ring at 5- C of isoxazole reduced the activity. When two methoxyl groups were present nevertheless of position in phenyl ring at 5-C of isoxazole exihibited anti-inflammatory activity. Presence of NO2 group at $4^{th}/5^{th}$ position of phenyl ring at 5-C of isoxazole exhibited anti-inflammatory activity only when substituent in phenyl ring at 3-C of isoxazole having oxygen containing substituent having hydroxyl/alkoxyl functional group.

The structure of compounds exhibited anti-inflammatory activity among tested 25 compounds are as follows;



4. Discussion

Isoxazole is a five membered heterocyclic compound having various pharmacological actions. Isoxazoles were reported to posses various biological activities. The reactive intermediate chalcones involved in their synthesis also exhibit wide range of biological activities. Ballesteros et al.(1995) reported that two synthetic 2-hydroxychalcones exerted topical antiinflammatory effects in mice. Lee et al.(2006) reported 2,4,6-tris(methoxy)chalcone to be an antiinflammatory compound that reduces nitric oxide (NO) production by inhibiting inducible NO synthase expression. The findings suggest that some chalcones may be promising antiinflammatory agents. In the light of these interesting biological activities, it appeared of interest to synthesize some new isoxazole derivatives and to evaluate their antiinflammatory activities. Inflammation is caused by the release of inflammatory mediators from tissues and migrating cells. Histamine, prostaglandin, leukotrienes, bradykinis, platlet activating factorand interleukin -1. Drug that inhibits cyclo-oxygenase and 5-lipoxygenase would expect to posses same anti inflammatory effect as steroids.

The synthesized isoxazole derivatives were screened for *in vivo* and *in vitro* anti inflammatory activity. The *in-vitro* anti-inflammatory has done by % inhibition of protein denaturation method. And *in-vivo* anti-inflammatory activity done by carrageenan induced paw edema and xylene induced ear edema of *Wister Albino rats a*nd compared with diclofenac sodium, which is a standard drug. As shown in figure1 the evaluation indicated that the 25 synthetic compounds showed antiinflammatory activity at a dose of 100 mg/kg administered orally 1 h before the inflammatory agent xylene. Among the synthesized compounds, L1, L4, L6, L8, L9, L15, and L19 Showed the highest ear inflammation inhibition rate. The Increase in Thickness and Inflammation induced by Xylene application is due to neutrophil accumulation which places a critical role in cutaneous inflammatory disease such as dermatitis and is related to pathological mechanism of disease.

In carrageenan induced paw edema method the paw volumes were recorded within 4 hr. interval time. Carrageenan is a complex polysaccharide Carrageenan works via the Bcl10, NF-κB, IκBα pathway to activate inflammation mediators. This pathway initially involves phosphorylation steps followed by nuclear translocation of phospho-NF-κB. This sets off transcription and translation of inflammatory biomarkers such as COX, NOS, IL-6 etc. Inflammation causes many effects. One of the effects is vasodilation of capillaries/blood vessels. Below the surface where carrageenan has been applied, inflammation thus causes dilation of the capillaries underneath the skin surface. Thus increased blood flow to the area. Manifest as swelling/redness of the affected area. The study indicated that compounds, L1, L4, L6, L8, L9, L15, and L19 had exhibited highly potent *in-vitro* and *in vivo* anti-inflammatory activity. Both showed significant anti-inflammatory activity when compared to standard diclofenac sodium. Other compounds exhibited less anti-inflammatory activity.

5. Conclusions

Presence of hydroxyl or alkoxyl functional group in 3-C substituted phenyl ring of Isoxazole exhibited moderate anti-inflammatory activity. But In the case of 5-C-phenyl ring position of substituents had influenze in exhibiting the proposed activity. Anti-inflammatory activity at molecular level has to be evaluated in detail and there is scope for further investigations.

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7. References:

1. Sathish N.K, Raviteja P, Ramakrishna S and Chethan I.A(2011), Synthesis, characterization and anti-inflammatory activity of some novel isoxazoles ,Der Pharmacia Lettre, 3(3): 378-382.

2. Habeeb, A. G.; Rao, P. N.; Knaus, E. E.(2001) Design and synthesis of 4,5-diphenyl-4-isoxazolines: novel inhibitors of cyclooxygenase-2 with analgesic and antiinflammatory activity., J. Med. Chem., 44, 3039.

3. S.pranaya,P venkata smitha,N.sreenivasa Reddy (2015)Evaluation of analgesic and anti-inflammatory activity of ventilage calyculata;International Journal of Life sciences, ,9(1),43-46.

4. Sangitha chandran (2012), Preliminary invitro assessment of anti-inflammatory property of Mikania scandens flower extract, Journal of advanced Pharmacy education and research, 2(1), 25-31.

5. Ferrero-Miliani L, Nielsen OH, Andersen PS, Girardin SE; Nielsen; Andersen; Girardin (2007), Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. Clin. Exp. Immunol, 147 (2), 227–235.

6. Dimmock, J. R.; Elias, D. W.; Beazely, M. A.; Kandepu, N. M., (1999) Bioactivities of chalcones. Curr. Med. Chem.; 6: 1125-1149.

7. Bandgar, B. P.; Gawande, S. S.; Bodade, R. G.; Totre, J. V.; Khobragade, C. N., (2010) Synthesis and biological evaluation of simple methoxylated chalcones as anticancer, anti-inflammatory and antioxidant agents. Bioorg. Med. Chem.; 18: 1364-1370.

8. Patil, C. B.; Mahajan, S. K.; Katti, S. A.,(2009) Chalcone: A Versatile Molecule. J. Pharm. Sci. Res. 1: 11-22.

9. Eddarir, S.; Cotelle, N.; Bakkour, Y.; Rolando, C., An efficient synthesis of chalcones based on the Suzuki reaction. Tetrahedron Lett.; 44: 5359-5363.

10. B R Dravyakar, D P Kawade, P B Khedaker and K P Bhushari. (2003)Design and synthesis of some new diphenyl amino isoxazolines as potent anti-inflammatory agent. Indian J Chem 2008; 47B:1559-67.

11. Haris J M and Spencer (1962). A modified pethismograph apparatus for recording volume changes in rat paw. J. pharm. Pharmacol.; 14:464-66.