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# Betulonic Acid- Potent Candidate for Lead Development for Various Chronic Diseases

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ABSTRACT

The present research work commenced with the collection of, **bhojpatra** (*Betula utilis*). *Betula utilis* (common name bhojpatra is known for its beneficial effects and medicinal values. Its potent derivative, (BetA, 3-oxo-20(29)-lupen-28-oic acid), (betulonic acid) is a pentacyclic lupine type triterpene, widely distributed throughout the plant kingdom. It has valuable biological properties such as antiviral,antitumor, anti-inflammatory, antimicrobial, hepatoprotective, as well as immunostimulant activities but its effectiveness is diminished by its poor water solubility. Efforts have been divided in this project work to surpass this disadvantage by obtaining complexes with hydrophilic substances such as amino acid alkyl esters and making the betulonic acid. In order to manifest its significant pharmacological actions we started with its extraction process which included various steps following reported methods such as hot and cold extraction, soxhlet extraction, column chromatographic techniques with different organic solvents such as methanol, ethanol, propanol, isopropanol, butanol, dichloromethane, etc. Initially, hojpatra was subjected to hot and cold extraction with above mentioned solvents and as a result, betulin was converted to betulonic acid using john's oxidising agent. On the other hand, amino acid alkyl esters were prepared using amino acids- glycine and arginine. Betulonic acid was conjugated with the prepared amino acid alkyl esters to obtain betulonic acid derivatives with desirable pharmacological actions. The synthesized betulonic acid derivatives were subjected to biological screening to evaluate their antimicrobial and anti-inflammatory effects.

Keywords: Betulonic acid, Bhojpatra, Anti-inflammatory, Antibacterial

## 1. INTRODUCTION

Lupane triterpenoids of plant origination, for example, betulin and betulinic and betulonic acids show an assortment of organic movement and are fascinating as beginning materials for synthetic and biocatalytic changes. (Tolstikov G. A., Flekhter O. B., E. E. Shults, Baltina L. A. & Tolstikov A. G, 2005) (Alakurtti S., Makela T., Koskimies S. & Yli-Kauhaluoma J, 2006)

Betulin is a phytochemical isolated from *Betula utilis* (**bhojpatra**, name in Sanskrit-Bhurja). The bark of birch trees consists of 10-15% of betulin and it can be easily isolated using simple extraction procedures. The family of this plant is **Betulaceae**.*Betula utilis*found shoot up beside moraines on all sides of bhojbasa in, nearabout the proboscis of the gangotri. It is a medium sized tree that grows upto a height of 20m. Its bark is glossy, reddish-white or whitish, with horizontal even and regular, lenticels. The shape of leaves are tapering, elliptical and irregularly serrate. The flowers are found to be monoecious which are pollinated by wind. The seeds are found to be slender and winged, Loamy and clay soils are suitable for the plant, it prefers fully dried soil and can even shoot-up in heavy clay soil. The favourable pH for the plant to shoot-up is acidic, neutral as well as alkaline soils. It can spring-up in partialdappled umbrage (light5) or in traunt shade. It possess antiseptic, odoriferous, antiflatulent and contraceptive effects. The phytoconstituents that the bark comprises are betulin, lupeol, acetyloheanolic acid, betulinic acid, a ketone-lupenone, β-sitosterol, methyl betultriterpenoid,. (Sharma Promila, Singh Saumya, Yadav Shivani & Thapliyal Ashish, 2012)

### *1.1. BETULONIC ACID—A POTENT LEADING BIOLOGICAL MOIETY*

Betu-lonic acid (BetA, 3-oxo-20(29)-lupen-28-oic acid), are pentacyclic triterpenes of the lupane type, which were isolated from birch bark. Betulonic acid and its derivatives can serve as interesting structural plan of action for the advancement of new therapeutic agents because of its high accessibility and a wide scope of natural activities.It has significant organic properties, for example, antiviral,antitumor, calming, antimicrobial, hepatoprotective, just as immunostimulant exercises.

It tends to be gotten rather simply from betulin by oxidation with chromium trioxide and betulin forms up to 30% of the dry weight of the extractive (the bark of birch trees). In this method preparation of betu-lonic acid merge two multi-step processes: (1) extraction ofbirch bark go after by concentration and purification of crudebetulin; and (2) oxidation of crude or pure betulin into betulonic acid go after by its purification. Mostly the production is fairly laborious and refined, including solvent type change at each resulting step of these processes. Hence, alcohols and halogenated hydrocarbons are regularly utilized for bark extraction is gotten by crystallization of the concentrates from methanolic and chloroformic solutions (Petrenko N.I.,Elantseva N. V., Petukhova V. Z., Shakirov M. M, Shul'ts E. E. & Tolstikov G. A. ,2002) **(**Kim Darrick S. H. L., Chen Zhidong, Nguyen van Tuyen, Pezzuto, John M., Qiu, Shengxiang & Lu, Zhi-Zhen,1997)

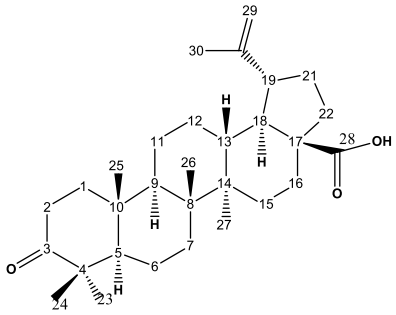



Fig.1- Chemical structure of betulonic acid

### *1.2. CONVENTIONAL UTILIZATION OF BETULA UTILIS*

The bark of this tree was employed many decades back in India as sheet of paper for scribbling long holy writings and contents of sanskrit and another written works. Bhurja is name given to bhojpatra in sanskrit, sharing an analogy with other Indo-European terminology. The bark is commonly used as a material for packing, for building ceiling etc. Leaves of this tree are effective in cure of infections in urinary tract and stones present in bladder. The wood is employed for building work and for fodder. Some parts of the plant have also been used in local traditional medicines for the treatment of the fungal growth (bhurja-granthi). (Sharma Promila, Singh Saumya, Yadav Shivani & Thapliyal Ashish, 2012)

Fig.2-Birch tree

## 2. MATERIAL AND METHODS

### *2.1. EXTRACTION AND ISOLATION OF BETULIN*

Presesnt research project work is based on the approach of semi-synthetic work on natural products. Lot of research work have been conducted on bhojpatra adopting both natural and synthetic approaches. But here, we have consolidated both natural as well as synthetic work. In this reference few reported extraction and isolation procedures were carried out which are as follows:

- Initially 15 gm of bhojpatra was boiled in methanol for about 10-15 minutes in iodine flask along with few porcelain chips. Then its solid part was separated from solvent part. The solvent front was then concentrated to the maximum and kept overnight in fridge. This procedure was

found to be the simplest and provides with maximum yield of betulin in impurified form.

- When impurified betulin obtained, its recrystallisation process was carried out with butanol or isopropanol and water.
- TLC of the synthesized compounds was carried out in order to ascertain its purity.

  **Mobile phase for TLC** : Ethyl acetate: benzene: formic acid (36:12:5)

  **Detecting agent:** Anisaldehyde(0.5mL) + $H_2SO_4$(1 mL 97%) + Glacial acetic acid (50 mL)

**Result** ⇨ Less violet spot- Betulin
More violet spot- Betulonic acid

- There were chances that certain percentage of quantity would have remained within the bhojpatra after this procedure. So, for its extraction to maximum extent soxhlet apparatus was employed along with various solvents such as chloroform, DMF, methanol, ethanol, propanol, isopropanol, butanol, etc. The resultant yield from different organic solvents was calculated and compared.
- Soxhlet procedure was used to raise the yield of the product. This is referred to as hot process extraction.
- Along with these procedures, cold extraction process was also attempted which involved dipping of bhojpatra for 3-4 days in ethyl acetate: ethanol: water (4.5: 4.5: 1) for 10 ml.

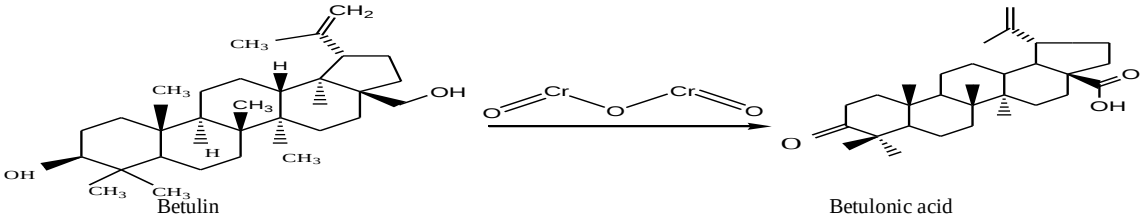
### *2.2. PREPARATION OF BETULONIC ACID*

#### *2.2.1 PREPARATION OF JONE'S REAGENT*

Jone's reagent was produced by dissolving chromium trioxide (70 gm, 0.70 moles) in 100 ml of water placed in a 500 ml beaker. The beaker was then kept in an ice bath. 18 ml of sulphuric acid (61 ml, 1.10 mole) and then 200 ml of water was further added on with constant manual stirring. The above mixture was then cooled to 0ºC - 5ºC.

#### *2.2.2 PREPARATION OF BETULIN TO BETULONIC ACID*

Jone's reagent was added drop by drop to a betulin (1 gm, 2.26 mmol) solution mixture in 50 ml acetone cooled to 0ºC,. Continuous stirring the above resulting mixture was done around 1.5 hrs at a temperature of 0º C, a further addition of 25 ml methanol was done, then for next 5 minutes the solution was stirred. 40 ml of water was further added. Vacuum was applied to remove the acetone and by using 40 ml of ethyl acetate the aqueous residue was extracted. The next step involved the separataion of aqueous layers from the ethyl acetate layer. Washing of ethyl acetate layer was done first with 20 ml of water followed by washing with 15 ml of brine. Magnesium sulphate was employed for drying ethyl acetate layer, filtered and further removal of ethyl acetate layer was done under vacuum. Then column chromatography of residue was carried out using 60-200 mesh silica gel employing petroleum ether/ ethyl acetate (4:1) to produce betulonic acid (770 mg), whose melting point was detected to be in the range 247º C- 249ºC. The reaction resulted in a 75% yield of betulonic acid.




Betulin Betulonic acid

Fig.3-Conversion of betulin to betulonic acid

### *2.3.GENERAL PROCEDURE FOR PREPARATION OF AMINO ACID ALKYL ESTER HYDROCHLORIDES DERIVATIVES OF BETULONIC ACID*

In a round bottom flask 0.1 mol amino acid was placed. Newly prepared distilled chlorotrimethylsilane (0.2 mol) was added steadily to this under continuous stirring with a magnetic stirrer. 100 ml of methanol was then added to the above mixture. The resulting solution was then mixed at 25ºC. The resulting mixture was concentrated with the help of rotary evaporator as the reaction completed, to yield the product amino acid ester hydrochloride.

.Following are the above mentioned method amino acid alkyl ester derivatives which were synthesized:

- Arginine methyl ester

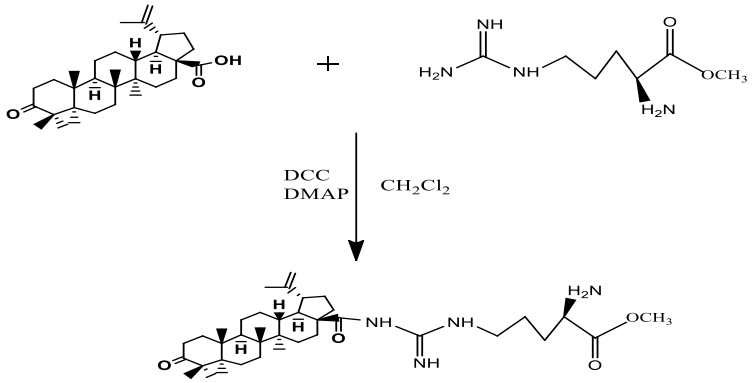
- Arginine ethyl ester
- Arginine propyl ester

Now, the next step consisted the chemical conjugation of synthesized amino acid alkyl ester derivatives with betulonic acid so as to derivitize biologically potent derivatives.

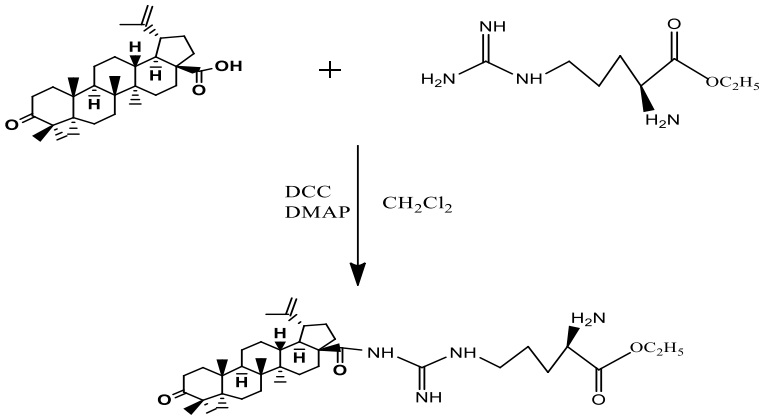
Dissolved Betulonic acid, amino acid alkyl ester and triethyl amine in tetrahydrofuran at room temperature. To this solution mixture added DCC and DMAP, stirred this for 48 hrs. Filtered the precipitate (Dicyclohexyl urea). Evaporated the filtrate to will remove THF. After evaporation, remaining was dissolved in ether/ethyl acetate (2:1). 100 ml water and HCl poured in separating funnel. Organic layer collected then dried on $MgSO_4$.

### *2.4.SYNTHETIC SCHEME FOR THE AMINO ACID ALKYL ESTER CONJUGATES OF BETULONIC ACID*

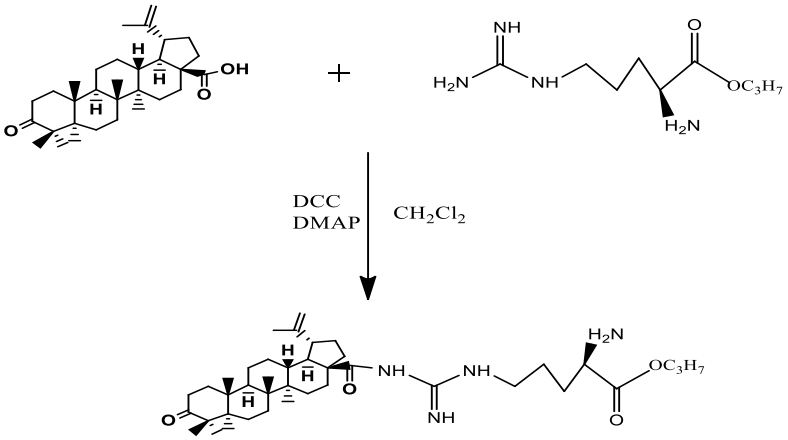
- Arginine methyl ester of betulonic acid




- Arginine ethyl ester of betulonic acid




- Arginine propyl ester of betulonic acid



## 3. BIOLOGICAL ACTIVITY

All the integrated compounds were screened for antimicrobial activity and anti-inflammation activity.

### *3.1. ANTIMICROBIAL ACTIVITY*

Paul Ehrlich elevated the concept of chemotherapy to medicate microbial diseases; he predicted the growth of chemical therapeuticals that would demolish microorganism without hurting the host. Pasteur established the actuality of antibiotics in 1877; air borne microscopic organisms restrained the improvement of Bacillus anthracis bacilli in urine. Sulpha medicaments came into significance in the last part of the 1930's; in 1929 Alexander Fleming found the 1st anti-biotic that was penicillin. Its initial clinical fundamentals were finished in 1940. Principal sulfonamide was Sulfa-pyridine. Waksman in 1940 and his comrate embraced a methodical hunt of acti-nomycetes although a wellspring of anti-biotics and locate streptomycinin 1944. This gathering of soil microbe's end up being a rich plcace of antibiotics and shortly antibiotic medication like Tetracyclines, Chloramphenicol, Erythromycin and numerous others followed. Many current antifungal have been available, two significant antibiotics; Amphotericin-B to manage with systemic mycosis and Griseofulvin to enhance attack on dermatophytes were presented close by 1960.

In the erstwhile 40 years significance has moved from looking new anti-infective producing antibiotics to creating semisynthetic subordinates of of more seasoned anti-microbials with considerably more engaging properties or clashing range action.

Anti-microbial medications are the greatest commitment of the current century to therapeutics. The importance is amplified in the developing countries where infective disease predominates. As a class they are one of the most oftentimes utilized as well as abused drugs.

#### *3.1.1 Tube dilution method:*

The antimicrobial agent dilutions will be favored in growth medium with the end goal that the medication concentration achieve the desired clinical significance range. An equivalent amount of broth consisting 105-106 microbes/ml will be then added on to every tube and also to a control tube in which no microbial antagonist is present. Noticeable turbidity will be inspected in the tubes for after an overnight incubation. This strategy is utilized for detecting susceptibility of microbes in liqud media.

#### *3.1.2 Agar diffusion method:*

In this procedure by employing pouring method Petri dishes of agar will be made. Inoculation of the agar will be done with microorganisms. In agar dilution method for the two; aerobes and anaerobes diverse antibiotic concentrations will be utilized in to an agar culture. For 24 hours at 37 ºC temperature the plates are brooded. The microbial antagonist disperses through the agar and forms an inhibition zone. The diameter of the zone can be determined and an assessment of the level of action of the microbial antagonist can be acquired.

#### *3.1.3 Media*

Nutrient agar media were utilized for the reason, which contains the constituents as introduced in table 1.

**Table 1 – Nutrient agar Media.**

| Constituents | Quantity Required |
|---|---|
| Peptic digest | 5gm/liter |
| Yeast Extract | 1.5gm/liter |
| Beef Extract | 1.5gm/liter |
| Sodium Chlolride | 5gm/liter |
| Agar | 15gm/liter |
| Distilled Water | 1 liter |

#### *3.1.4 Experimental procedure:*

Agar-diffusion method was utilized for the determination of preliminary bacterial antagonist activities. The agar well diffusion test was carried out using nutrient agar medium, according to the system set out by Magaldi *et al.* 2004 and at 15 lbs pressure (121℃) this agar medium was autoclaved for 15minutes and afterward it was cooled instantly to 50- 55℃ in ice-bath. This medium was filled in petridishes to a static 4 mm depth; this is proportional to round about 40mL in a 90mm plate. The culture was then inoculated on the surface of medium after the medium had solidified. These were performed in a laminar air flow. The germ free swab was utilized on the outer surface of the nutrient agar culture to guarantee a uniform distribution. Then the petridishes were settled for few minutes to ensure abundant moisture absorption. Germ free plug borer (7mm)was utilized for making agar wells, and the concentrations of the 25, 50, 75, 100 and 200 µg/ml of the diluted stock solutions were set in each wells(Indian Pharmacopoeia, 1996).

The level of inhibition can be determined utilizing the equation :

$$\% \text{ Inhibition} = \frac{\text{I (diameter of inhibition zone in mm)}}{\text{90(diameter of Petri-plates in mm)}}$$

***3.1.5 Test strains***

For the current work, effectiveness of the test compounds was resolved against following bacterial strains:

Gram +ve bacterial strains-

- *Bacillus pumilus*
- *Staphylococcus aureus*

Gram -ve bacterial strains-

- *Escherichia coli*
- *Klebsiella pneumonia*

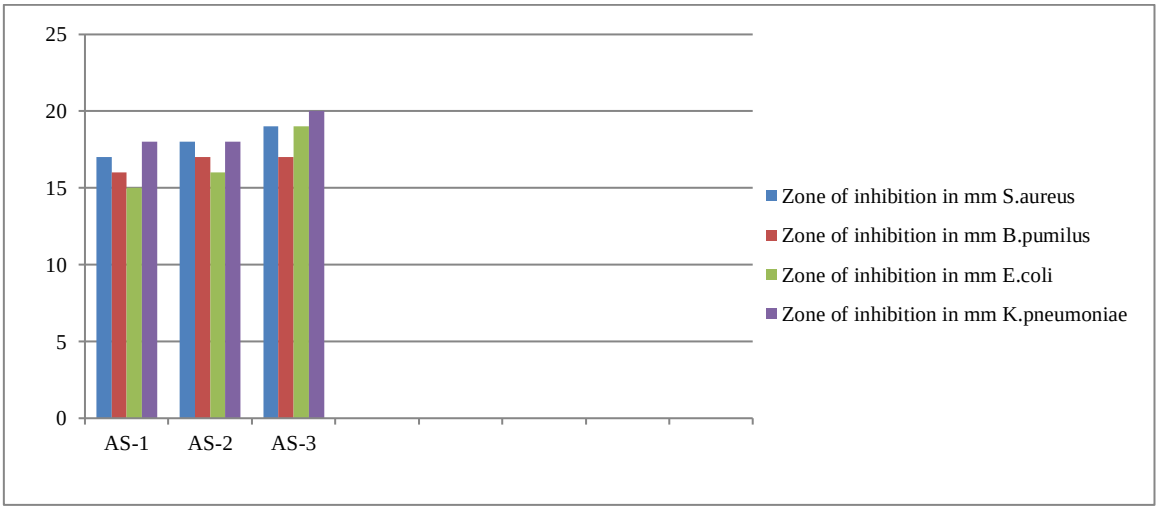



Fig.4- Antibacterial activity of synthesized compound

***3.2 Anti-inflammatory activity***

Inflammation antagonist action was completed via carrageenan included paw oedema process in albino rats (120-160g). The antiinflammatory evaluations of the incorporated compounds were executed using reported procedures **(Winter et al, (1962)**. Diclofenac sodium was utilized as standard medication for correlation. Drugs and tested compounds were administered oral route by making 1% CMC suspension. Newely prepared aqueous suspension of carrageenan (1% w/v, 0.1 mL) was infused into the paw of each rat.

The investigation were done on the healthy adult albino rats (male/female) weighing between 120-160 g using carrageenan induced rat paw oedema method. They were fed on standard pellet diet and water.

***3.2.1 Standard drug:*** Diclofenac sodium (10 mg/kg)

***3.2.2 Equipment-*** Mercury displacement (plethysmograph).

***3.3.3 Purpose and Rationale:***

Among the strategies utilized for screening of inflammation antagonists, one of the most usually employed methods depends on the capacity of such specialists to restrain the edema build in the rear paw of the rodent after infusion of phlogistic agents.Brewer's yeast, formaldehydes, dextan, egg albumin, kaolin, aerosol, sulphated polysaccharides like carrageenan or naphthoyl heparamine have been utilized as phlogistic agents (irritants)

This impact can be quantified in few different ways. Typically the volume of the administered paw is estimated in advance and after implementation of the aggravation and the paw volume by simple and less exact and by increasingly sophisticated electronically devised techniques. The estimation of the assessment is rarely dependent on the apparatus but much more on the irritant being selected.

Few irritant incite just a short enduring swelling while different irritant prompt the paw edema to offer more than 24 hours.

***3.3.4 Procedure***

- The animals were partitioned into groups, each group consist 6 rats. One group of animals designated to control and another one is for

standard drug (Diclofenac) respectively. Remaining groups dispensed to the test compounds.

- Diclofenac and sample compounds were administered by oral route by preparing 1% CMC suspension to groups (standard, control and test compound) respectively
- After 30 minutes, 0.1 mL of 1% newly prearranged suspension of carrageenan in 0.9% solution of NaCl was subcutaneously infused in the paw and amount was estimated.
- The foot volume was estimated again at 2 hrs and 4 hrs, the mean increment in the paw volume in one and all group was determined.
- By using water plethysmometer apparatus The paw volume was estimated
- The variation in volume gave the measure of edema created.
- The percentage of inhibition value determined by following equation:

  % Antiinflammatory action = $[1- D_t/D_c]*100$

  $D_t$ =Paw volumes of oedema in test.

  $D_{c=}$=Paw volumes of oedema in control.

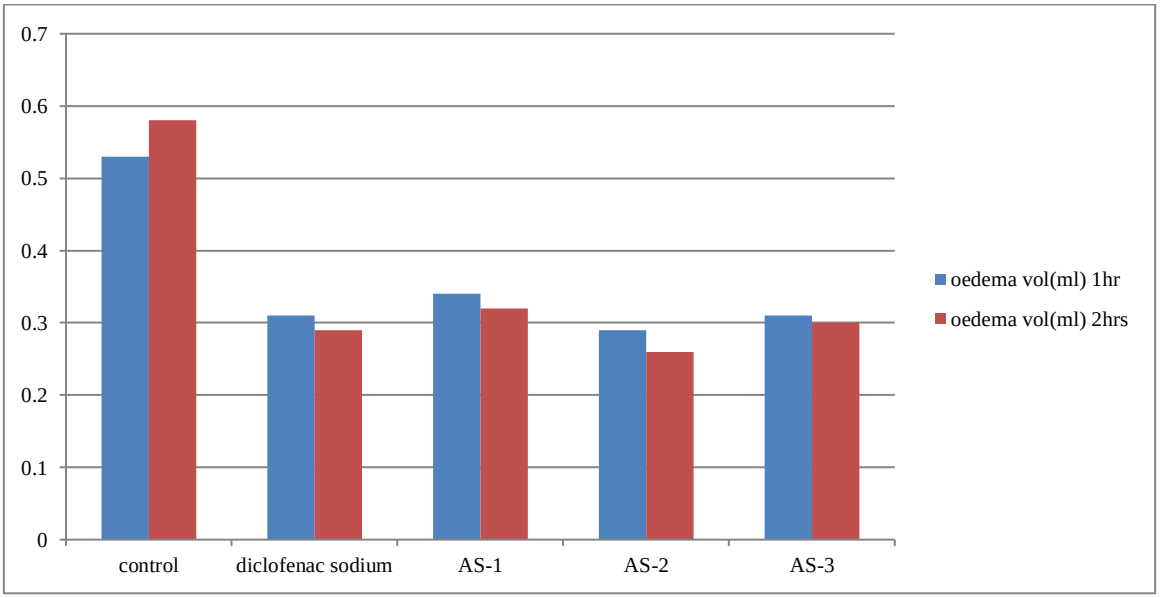



Fig.5- Anti-inflammatory screening (% inhibition) of synthesized compopunds.

## 4. RESULTS AND DISCUSSION

### *4.1. Results of in-vitroAntibacterial screening*

The *in-vitro* antibacterial activities of newly synthesized compounds (AS-1 to AS-5) were carried out by Agar Diffusuion Method counter the micro organisms viz. gram positive (*Bacillus pumilus*, *Staphyococcus aureus*) gram negative (*Escherichia coli*, *Kl*ebsiella pneumonia). All three derivatives were screened for antibacterial activities at 1000µg/mL concentrations.

The inhibition zone (in mm) was estimated for each compound accompanying ciprofloxacin as standard drug and results were presented graphically in **fig.4.**

Results demonstrated that compound **AS-1** showed maximum activity (inhibition zone in mm) against *Klebsiella pneumonia*, least action against, *Escherichia coli.*

Compound **AS-2** showed maximum activity (inhibition zone in mm) against *Klebsiella pneumonia*, *Staphylococcus aureus* and minimal activity against *Escherichia coli.*

Compound **AS-3** showed maximum action (inhibition zone in mm) against *Klebsiella pneumonia* and minimum activity against *Bacillus pumilus.*

### *4.2. Results of anti-inflammatory screening*

This result showed that the compounds possessing electron withdrawing group increase the action. Compound **AS-1 and AS-2** revealed remarkable anti-inflammatory activity after both 1 hrs and 2 hrs. Diclofenac sodium (10 mg/Kg) was used as standard.

## SUMMARY AND CONCLUSION

The basic purpose of the present research work seems to be served and appreciably fulfilled as encouraging results have been obtained both in terms of establishment of structural features as well as the spectrum of the biological activities of the synthesized compounds. Results of the biological screening have revealed that the compounds AS-1 and AS-2 were having moderate to good anti-inflammatory effect after 1hr & 2hrs at a dose of 10mg/Kg. The results of the antibacterial study revealed that all three compounds (AS-1, AS-2, AS-3) exhibited medium to good antibacterial activity when juxtaposed with the standard drug.

In nutshell, it may be concluded that the present research work may suggest new ideas and open new vistas for further research in the field of drug discovery and development particularly as far as structural manipulations in the synthetic medicinal chemistry is concerned.

## ACKNOWLEDGMENTS

God is our refuge and strength, a very present help when we need it the most I thank him for guiding me through rough and torn paths, for always being less whisper away. We pray that he will always bless us, always show us the right path. We worship his love and care through which he has showered achievement and happiness upon us.

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