

## Full Length Research Paper

# Nucleoside triphosphate diphosphohydrolases (NTPDase) inhibitory activity of some medicinal plants.

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Nucleoside triphosphate diphosphohydrolases (NTPDases, EC 3.6.1.5) are well characterized ectoenzymes which are involved in the hydrolysis of ATP and ADP resulting in the formation of nucleoside monophosphates and orthophosphate. By hydrolyzing circulating ATP to ADP to AMP, NTPDases play a crucial role in preventing the activation of platelets and their aggregation. NTPDase inhibitors therefore are currently target for various cardiovascular diseases and some types of cancer. In search for new enzyme inhibitors of therapeutic importance, the present study was conducted to find out the inhibitors of NTPDase from methanolic extracts of 50 medicinal plants used in folk medicine. Data shows that only 6 plants exhibited anti-NTPDase activity above 50% with crude enzyme preparation of chicken liver at 125 µg plant extract. These plants included, *Cymbopogon jawarancusa*, (51.16±4.81%), *Asparagus officinalis* (58.24±0.93%), *Tribulus terrestris* (50.84±6.19%), *Rubia cordizolla* (53.11±3.49%), *Echimops echimatus* (51.48±6.29%) *Portulaca oleracea* (52.79±0.10%). These studies are preliminary findings and further work is required to explore these plants using purified enzyme preparations.

**Keywords:** Nucleoside triphosphate diphosphohydrolases, NTPDases, medicinal plants.

## INTRODUCTION

Nucleoside triphosphate diphosphohydrolases (EC 3.6.1.5, NTPDases) are well characterized ectoenzymes in the central nervous system (Rocha et al., 1990). The importance of NTPDase in the tissues is related closely to the presence of urinoreceptors on the cells (Beaudoin et al., 1997). This enzyme hydrolyzes ATP and ADP resulting in the formation of nucleoside monophosphates:



Its maximal catalytic activity is adapted to the extra cellular ion environment and requires the presence of divalent cations, such as Ca or Mg (Zimmerman, 2001). NTPDases are localized on the surface of many cell types including endothelial cells lining of blood vessels. By converting circulating ADP to AMP, it plays a crucial role in preventing the activation of platelets and their aggregation (Gendron et al., 2002).

## Classification of NTPDase enzyme

Different families of these ecto-enzymes have been identified molecularly and functionally including the ecto-nucleoside triphosphate diphosphohydrolase (E-

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NTPDase) family. NTPDases are characterized in eight members, named NTPDase-1 to NTPDase-8. These members share highly conserved domains called apyrase-conserved regions (ACR). ACRs are probably involved in the catalytic activity and structural integrity of the NTPDase. Four members of this family, NTPDases 1, 2, 3, and 8 are anchored in the plasma membrane with catalytic sites facing the extra cellular space and are implicated in the control of nucleotide levels at the cell surface. While NTPDases 4–7 mainly lie in the membranes of intracellular organelles and are not clearly involved in extra cellular nucleotide signaling (Zimmerman et al., 2000; Zimmerman, 2001; Bigonnesse et al., 2004; Kukulski et al., 2005).

### Role and inhibition of NTPDases

NTPDases are powerful natural anti-platelet agents that prevent the initiation of the coagulation process and reduce thrombogenicity. Moreover, perfusion of this enzyme appears to prevent the colonization of the ischemic tissues by macrophages and neutrophils after thrombosis, thereby reducing considerably their necrosis. They are directly involved in the immune response. NTPDases are agonist of T-lymphocyte proliferation and humoral response (Gendron et al., 2002).

The NTPDase inhibitors known so far are derived from three different chemical classes (Kennedy, 1990):

1. Nucleotides and their analogues.
2. Sulfonated dyes such as Reactive Blue 2.
3. Suramin and its derivatives.

NTPDase inhibitors have potential for the treatment of cancer and diseases of the immune system. Inhibition of NTPDases may increase the immune response towards viral or bacterial infections. NTPDase inhibitors are able to inhibit T-lymphocyte proliferation and humoral response. NTPDase inhibitors are used in leukemia, lymphoma, autoimmune disorders, such as rheumatoid arthritis and psoriasis (Zimmermann et al, 2000). NTPDase inhibitors known so far are derived from three different chemical classes (Kennedy et al., 1996), that is, nucleotides and their analogues, sulfonated dyes such as Reactive Blue 2 and suramin and its derivatives (Crack et al., 1995). NTPDase inhibitors of the class natural products have not been explored. A study has been reported by De-Silva et al., (2006) in *Casuarina sylvestris*. Therefore, the present studies were carried out to screen methanolic extracts of plants possessing anti-NTPDase activity.

## MATERIALS AND METHODS

### Plant material

Plants or their parts were purchased from the local market. They

were identified and authenticated by Dr M. Arshad, Cholistan Institute of Desert Studies (CIDS), The Islamia University of Bahawalpur.

### Preparation of plant extracts

Plants were dried under shade and ground with electric grinder to fine powder. Ground powder (100 g) was soaked in 400 ml methanol for two weeks with occasional shaking. The soaked material was filtered through miracloth. This process was repeated 3x with 200 ml methanol to extract maximum material and all contents were pooled. The solvent was evaporated at room temperature or under vacuum with rotary evaporator. The residue was collected in small glass bottles and stored at 4°C. Plant methanolic residue was weighed and dissolved in 100 mM Tris buffer pH 7.4, which dissolved maximum residue and this stock solution of 1 mg/ml was used for the determination of enzyme activities. This procedure was done to avoid denaturation of enzymes due to organic solvent during the assay and get reproducible data.

### Preparation of enzyme source

Fresh chicken liver (1 g) was purchased from the local market, washed with 50 mM Tris-HCl buffer pH 7.4 and homogenized in 10 ml extraction buffer (50 mM Tris-HCl buffer pH 7.4, 1mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 0.32M sucrose) in a homogenizer for 15 sec each after 10 sec intervals (De Silva et al., 2006). Test tube was placed in ice bucket to avoid heating. Contents were filtered through 3 layers of miracloth and centrifuged at 15,000 rpm for 15 min at 4°C (Model 1710, Kubota, Japan). The supernatant was used as a source of enzyme. Enzyme source was made fresh everyday and used within 4 hours. Protein was determined by Bradford method (Bradford, 1976) and 40-60 µg protein (10 µl) was used per assay.

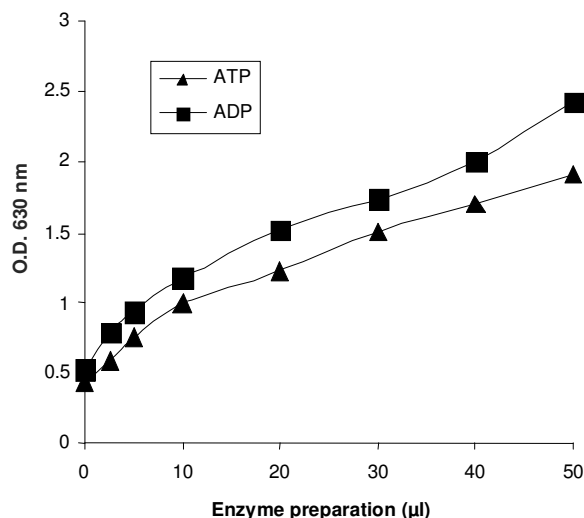
### Determination of NTPDase activity

The total reaction mixture (200 µl) consisted of 160 µl 500 mM Tris-HCl buffer, pH 7.4, with/without 5-20 µl (25-100 µg) plant extract followed by the addition of 10 µl fresh enzyme preparation (40-60 µg protein) from chicken liver homogenate. The contents were mixed and preincubated for 10 min at 30°C. The reaction was initiated by the addition of 10 µl of 100 mM ATP or ADP (20 mM working strength). After 30 min incubation at 30°C, the reaction was stopped by the addition of 200 µl 10% TCA (trichloroacetic acid). An aliquot of 20 µl was taken in 96-well plate and 200 µl malachite green reagent was added per well. Inorganic phosphate was measured at 630 nm within 4-7 min. Control experiments were carried out to correct for non-enzymatic hydrolysis by adding enzyme after the addition of TCA. All experiments were carried out with their respective controls.

### Preparation of color reagent

The method of (Chan et al., 1986) was used for the preparation of malachite green reagent in order to determine the released of phosphate during the assay. Reagent was prepared as follows:

|                    |                 |         |
|--------------------|-----------------|---------|
| Malachite green    | 0.812 g/1000 ml | 2 Parts |
| Ammonium molybdate | 22.8 g/400 ml   | 1 Part  |
| Polyvinyl alcohol  | 9.20 g/400 ml   | 1 Part  |
| Deionized water    |                 | 2 Parts |



**Figure 1.** Effect of varying concentrations of NTPDase on the rate of reaction.

Malachite green with few drops of concentrated HCl (12N) in 500 ml deionized water was taken and stirred on magnetic stirrer for 30 min until complete solubilisation. Its volume was made up to 1000 ml. Ammonium molybdate was added in 400 ml HCl (6N) and dissolved. Polyvinyl alcohol was dissolved in 400 ml deionised water by continuous heating. 2 parts malachite green, 2 parts deionised water, 1 part ammonium molybdate and 1 part polyvinyl alcohol were mixed and the final reagent was stored at room temperature (Chan et al., 1986).

#### **Preparation of other reagents for NTPDase assay**

|                 |         |
|-----------------|---------|
| Tris-HCl pH 7.4 | 500 mM  |
| TCA             | 10%     |
| ATP/ADP         | 100 mM. |

#### **NTPDase enzyme assay**

The 200 µl reaction mixture consisted of 160 µl 500 mM Tris-HCl buffer pH 7.4, with/without 5 to 20 µl (25 to 100 µg) plant extract followed by the addition of 10 µl enzyme (40 to 60 µg protein) from chicken liver homogenate. The contents were mixed and preincubated for 10 min at 30°C. The reaction was initiated by the addition of 10 µl of 100 mM ATP or ADP (20 mM working strength). After 30 min incubation at 30°C, the reaction was stopped by the addition of 200 µl 10% TCA. An aliquot of 20 µl was taken in 96-well plate and 200 µl malachite green reagent was added per well. Inorganic phosphate was measured at 630 nm within 4 to 7 min. Control experiments were carried out to correct for non-enzymatic hydrolysis by adding enzyme after the addition of TCA. All experiments were carried out with their respective controls.

#### **Determination of other phosphate hydrolyzing enzymes**

##### **Determination of phosphodiesterase activity**

Phosphodiesterase (EC 3.1.4.17) activity was determined in the crude enzyme extract by modified method of Sulkowski and Laskowski (1971). The 200 µl assay mixture contains 10 µl (5 mM

PNP) bis-p-nitrophenyl phosphate, 5 µl (0.25 mM) MgCl<sub>2</sub> and 100 mM Tris-HCl 7.4. Contents were preincubated at 30°C, 0.25 mM p-nitrophenyl phosphate (PNP) was added and then incubated for 30 min at 30°C. The reaction was stopped by 10% TCA. Aliquots of 20 µl were taken in the 96 well plate from total assay mixture and 200 µl malachite green reagent was added. The absorbance was read at 630 nm (Chan et al., 1986). Alternatively, the reaction was stopped by 0.1N NaOH and p-nitrophenol produced was measured at 400 nm.

##### **Determination of 5'-Nucleotidase activity**

The 5'-nucleotidase (EC 3.1.3.5) activity was determined by the modified method of Sinsheimer and Koerner (1952). The 200 µl reaction mixture contained 500 mM Tris-HCl buffer pH 7.4, 10 mM MgCl<sub>2</sub> and 100 mM AMP. 10 µl (40 to 60 µg protein) crude enzyme preparation was added. The mixture was incubated after the addition of 10 µl AMP at 30°C. An aliquote of 20 µl was taken in the 96 well plate from total assay mixture and 200 µl malachite green reagent was added. The liberated phosphate was determined at 630 nm.

##### **Determination of alkaline phosphatase activity**

The alkaline phosphatase (EC 3.1.3.1) activity was determined according to the spectrophotometer method of (Hausamen et al., 1967) at 25°C using (10 µl) p-nitrophenyl phosphate (PNP) as substrate. The 200 µl assay mixture contained 0.9M diethanolamine and 10 µl (40 to 60 µg) of chicken liver extract as source of enzyme. The control was also run under the same conditions and with the same components except the enzyme was omitted. The reaction was stopped by the addition of 10% TCA. Enzyme was added in control after TCA. The liberated phosphate was determined according to Chan et al. (1986). Alternatively, the reaction was stopped by the addition of 0.1N NaOH and O.D. read at 400 nm.

## **RESULTS**

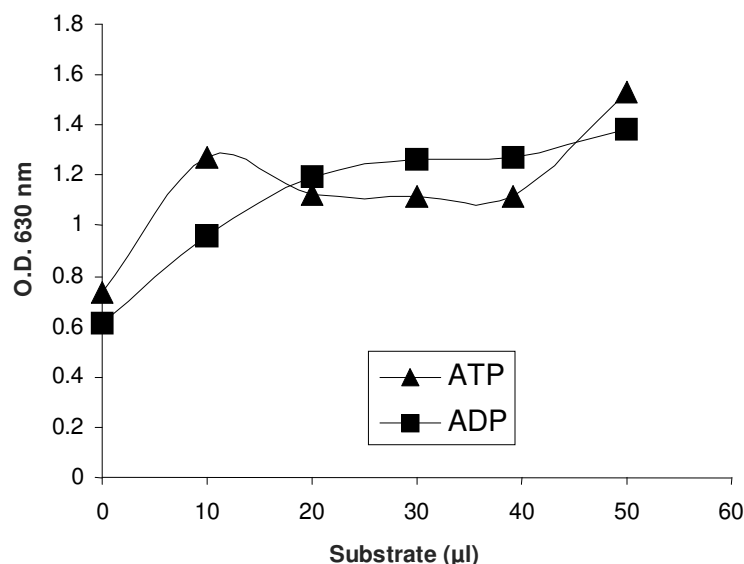
### **Inhibition studies of NTPDase**

NTPDase activity was determined using crude enzyme preparation of fresh chicken liver as given in the Materials and Methods. Protein contents were determined by Bradford method. Firstly, the method was standardized and then the inhibition studies were carried out (Figure 1).

### **Standardization of NTPDase method and plant inhibitory activity**

#### **Determination of the enzyme concentration per assay**

Various concentrations of enzyme preparation were used employing 10 µl of 100 mM ATP and ADP substrates. Results are shown in Figure 2. It clearly indicates that with increase in enzyme concentration using ATP or ADP as substrate, there was significant increase in the rate of reaction and formation of orthophosphate as determined by the method of Chan et al. (1986).



**Figure 2.** Effect of varying concentrations of substrates on the rate of reaction.

**Table 1.** NTPDase inhibition activity of 125 μg methanolic extracts of plants (n=3, mean±sem).

| Plant                        | Family     | Plant parts inhibition | Percent    |
|------------------------------|------------|------------------------|------------|
| <i>Asparagus officinalis</i> | Lilliaceae | seed                   | 58.24±0.93 |
| <i>Rubia cordizolla</i>      | Asteraceae | whole plant            | 53.11±3.49 |
| <i>Echimops echimatus</i>    | Asteraceae | whole plant            | 51.48±6.29 |
| <i>Portulaca oleracea</i>    | Apiaceae   | seed                   | 52.79±0.10 |

### Determination of the substrate concentration per assay

Various concentrations of ATP and ADP (100 mM stock solution in Tris buffer pH 7.4) were used against 10 μl enzyme preparation containing 40 to 60 μg protein. The rate of reaction was determined by change in absorbance of the liberated orthophosphate as determined by Malachite green reagent method.

Results showed more than 50% anti-NTPDase activity at 125 μg plant extract using ATP as substrate: *C. jawarancusa*, (51.16±4.81), *A. officinalis* (58.24±0.93), *T. Terrestris* (50.84±6.19), *R. cordifolia* (53.11±3.49), *E. echimatus* (51.48±6.29) *P. oleracea* (52.79±0.10). Crude enzyme source was also checked for the determination of the activities of other phosphate hydrolyzing enzymes, like phosphodioesterase, alkaline phosphatase, acid phosphatase and nucleotidase and the contribution they made in the assay of NTPDase (data not shown). There is a need to prepare a dose dependent enzyme inhibition study with the purified enzyme preparation and isolation

and purification of active constituents from the said plants. A similar study has been reported by De-Silva (2006) in *Casearia sylvestris*.

### Activities of other phosphate hydrolyzing enzymes

Crude enzyme source was checked for the determination of the activities of other phosphate hydrolyzing enzymes, the contribution they made in the assay of NTPDase as given in the Table 1.

### DISCUSSION

Today, enzymes are the targets for new drug discovery, it involves the interaction with substrate to form the product. The inhibitor will also interact with the enzyme and decrease the rate of reaction. The enzyme inhibition assays have prompted us to carry out a primary

screening of the methanolic extracts of plants commonly used in Pakistan. Therefore, these studies were conducted to investigate the presence of some therapeutically important drugs present in some medicinally used plants that have the ability to inhibit some particular enzyme and to what extent it is present. Methanolic extracts of more than 70 plants were collected. These extracts were used in the initial screening for any enzyme inhibitors. As far as the activity of anti-NTPDase is concerned, results showed more than 50% anti-NTPDase activity at 125 µg plant extract using ATP as substrate: *C. jawarancusa*, (51.16±4.81), *A. officinalis* (58.24±0.93), *T. Terrestris* (50.84±6.19), *R. cordifolia* (53.11±3.49), *E. echinatus* (51.48±6.29) *P. oleracea* (52.79±0.10).

## Conclusion

In summary, six plants extracts possess moderate levels of inhibitory activity against NTPDase and all other plant extracts possess more or less anti-NTPDase activity. Plant extracts should be further subjected to chromatographic separations and phytochemical analyses and the purified NTPDase should be used to carry out more inhibition studies.

## REFERENCES

- Aaron J, Marcus M, Johan B, Joan HF, Drosopoulos KE, Olson N, Islam DJ, Pinsky R, Levi S, Thromb H (2005). Semin Thromb. Hemost., 31: 234-246.
- Atkinson B, Dwyer K, Enjoji K, Robson SC (2006). Blood Cells Molecules and Diseases, 36: 217-222.
- Beaudoin AR, Grondin G, Sevigny J (1997). Plenum Press, New York, pp. 33-39.
- Bigonnesse F, Levesque SA, Kukulsiki F, Lecka J, Robson SC, Fernandes MJ (2004). Sevigny J. Biochem., 43: 5511-5519.
- Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem., 72: 248-254.
- Chan KM, Delfert D, Junger KD (1986). A direct colorimetric assay for  $\text{Ca}^{2+}$  Stimulated ATPase activity. Anal. Biochem., 157: 375-380.
- Crack BE, Pollard CE, Beukers MW, Roberts SM, Hunt SF, Ingall AH, (1995). Pharmacological and biochemical analysis of FPL 67156, a novel, selective inhibitor of ecto-ATPase. Br. J. Pharmacol., 114: 475-481.
- Drosopoulos JH, Broekman MJ, Islam N, Maliszewski CR, Gayle RB, Marcus AJ (2000). Site-directed mutagenesis of human endothelial cell ecto-ADPase/soluble CD39: requirement of glutamate 174 and serine 218 for enzyme activity and inhibition of platelet recruitment. Biochem., 39: 6936-6943.
- Da-Silva AC, Balz D, de-Souza JB, Morsch VM, Correa MC, Zanetti GD, Manfron MP, Schetinger MR (2006). Inhibition of NTPDase, 5'-nucleotidase,  $\text{Na}^+/\text{K}^+$  ATPase and acetylcholinesterase activities by subchronic treatment with *Casearia sylvestris* Phytomed., 13(7): 509-514.
- Gendron FP, Benrezzak O, Krugh BW, Kong Q, Weisman GA, Beaudoin AR (2002). Purine signaling and potential new therapeutic approach: possible outcomes of NTPDase inhibition. Curr. Drug Target, 3: 229-245.
- Hiroaki H, Ebata T, Uesugi S, Fujii S (1990). Base recognition mechanism of bleomycin: solution structure of d(GGGGAGCTCCCC)2 based on 1H-NMR and restrained molecular dynamics. Faculty of Pharmaceutical Sciences, Osaka University, Japan. Nucleic Acids Symp. Ser., 22: 53-54.
- Kennedy C, Westfall TD, Sneddon P (1996). Modulation of purinergic neurotransmission by ecto-ATPase Semin Neurosci., 8: 195-199.
- Rocha JBT, Mello CF, Sarkis JJF, Dias RD (1990). Undernutrition during the preweaning period changes calcium ATPase and ADPase activity of synaptosomal fraction of weaning rats Br. J. Nutr., 63: 273-283.
- Sinsheimer RL, Koerner JF (1952). The 5'-nucleotidase activity determined by the modified method J. Biol. Chem., 198: 293.
- Sulkowski E, Laskowski M (1974). Sr: Venom Exonuclease (Phosphodiesterase) Immobilized on Concanavalin-A-Sephadex, Biochem. Biophys. Res. Commun., 57: 463.
- Zimmermann H., (2001). Ectonucleotidase some recent development a note on nomenclature. Drug Dev. Res., 52: 44-56.
- Zimmermann H, Beaudoin AR, Bollen M (2000). Proposed nomenclature for two novel nucleotide hydrolyzing enzyme families expressed on the cell surface. In: vanDuffel L, Lemmens R (eds) Ecto-ATPases and related ectonucleotidases, Shaker BV, Maastricht, pp. 1-8.
- Kukulski F, Lévesque SA, Lavoie ÉG (2005). Comparative hydrolysis of P2 receptor agonists by NTPDase 1, 2, 3 and 8. Purinergic Signalling, 1: 193-204.