A Comparative Analyses of Dexamethasone and Neem Leaf Extract for Treating Cigarette-Smoke Induced Liver Injury in Swiss Mice

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Research Article

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Abstract

Cigarette smoke induces cytokines and lipid peroxidation in liver cells contributing to one of the causes for mortality across the globe. Dexamethasone is widely used for medication yet it decreases the levels of glutathione peroxidase and superoxide dismutase in macrophages. Therefore, present study focused on the comparison between dexamethasone and *Azadirachta indica* leaf extract for treating hepato-injury caused by cigarette smoke in mice. The phytochemical screening of *A. indica* ethanolic leaf extract revealed the abundance of sterols, proteins, alkaloids, flavonoids and diterpenes that could have antioxidant and anti-inflammatory activities. In this context, Swiss mice were treated with cigarette smoke, dexamethasone and *A. indica* leaf extract. Dexamethasone treated mice exclusively resulted in mortality rate of 20%. These mice expressed elevated inflammatory cells with higher levels of liver markers, alanine aminotransferase (ALT; 56.00 IU/L) and aspartate aminotransferase (AST; 64.90 IU/L). Moreover, these mice revealed the prevalence of relative oxygen species (ROS) at 32500 RFU and eosinophil peroxidase (EPO) level at 1.97 absorbance units with declined superoxide dismutase (SOD) levels of 4.3 n kat/mg. Whereas, tissue recovery was spotted in the mice treated with *A. Indica* leaf extract with highly declined levels of liver markers, AST (10.20 IU/L) and AST (15.90 IU/L). Furthermore, these mice resulted in lesser amount of ROS at 21900 RFU and EPO levels at 1.08 absorbance unit with increased SOD levels of 7.17 n kat/mg. This is the first report demonstrating *in vivo* effects of *Azadirachta indica* ethanolic leaf extract in treating cigarette smoke induced hepatic chronic inflammation in animal model which gave better results when compared to dexamethasone.

Introduction

Inflammation is a primary defensive response expressed by living cells against the noxious intracellular and extracellular agents. Every so often, these cellular responses continue to remain constant for a long, developing into chronic diseases which require potent pharmacological treatments (Demir 2020). However, these treatments may have adverse effects and therefore alternative natural therapies are acknowledged to cure long term illness. Various herbs and plants are persistently in use since the Vedic era for the treatment of severe diseases including blood and urine infections, myocardial infarction, kidney failure, lung diseases, and many more (Sen et al. 2017).

There are several reasons beholding the cause for liver damage such as asthma, diabetes, alcohol consumption, chronic obstructive diseases (Yong et al. 2020). Smoking causes architectural variations leading to the malfunctioning of the organ by increasing oxidative stress, chronic inflammation in tissues and enhances the production of stress kinases, inflammatory cytokines, and lipid peroxidation which ultimately injures liver tissues. Cigarette smoke alters the activity of NF-κB, a redox-sensitive transcription factor, playing a significant role in oxidation regulation and inflammation. It modifies the NF-κB expression by increasing the expression of other regulatory genes in non-parenchymal hepatocytes. Moreover, cigarette smoking causes oxidative injury by reducing superoxide dismutase (SOD) activity in addition to elevation in eosinophil peroxidase (EPO) and serum aminotransferases activity (Ponist et al. 2019). Under these circumstances, it becomes obligatory to diagnose liver damage which is facilitated by
the examination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels (liver injury markers) since RBCs are rich in AST that leaks into plasma before haemolysis (Raja et al. 2011). Dexamethasone is a commercial anti-inflammatory corticosteroid widely used for the treatment of chronic obstructive pulmonary disease (COPD), but inspite of that its long-term use or high doses has been reported to cause various harmful side-effects including insulin resistance, hyperglycemia and weight change (Malkawi et al. 2018). As a consequence of this, there is a requirement of substitutes that could potentially be effective against aforesaid diseases with limited side-effects. Several plants have been exploited extensively in traditional Indian culture (Ayurveda) which are of medicinal value and have been used to treat a number of diseases with least side-effects (Balunas et al. 2005). *Azadirachta indica* (neem) is native to Indian sub-continent and has been used widely for centuries as medicine to cure number of diseases because of its antioxidant, antimicrobial, anti-inflammatory, antigastric ulcer, antipyretic, hypoglycaemic and anti-tumor activities (Eid et al. 2017). There are several bioactive compounds present in leaf extract of neem such as tannins, sterols, alkaloids, flavonoids, diterpenes, and proteins (Benisheik et al. 2019). Research has shown that *A. indica* extract has safe nature that set the basis for its use over thousands of years. Flavonoids reported to have antioxidant and free radical scavenging activity (Kanagasanthosh et al. 2015) and with regard to this, it is assumed that the phytochemicals present in neem leaf extract probably are responsible for its potential anti-inflammatory activity. This study was designed to examine the effect of ethanolic leaf extract of neem in comparison to dexamethasone against hepato-injury caused by cigarette smoke in mice.

**Materials And Methods**

**Plant Materials**

The fresh leaves of *Azadirachta indica* were collected from BHU campus and then identified by Prof. NK Dubey (with a voucher number of *Melia. 2019/1*), Department of Botany, Institute of Science, Banaras Hindu University, Varanasi.

**Preparation of Azadirachta indica ethanolic extract**

Plant leaves were grind using mortar-pestle and subjected to alcoholic extraction by soxhlet apparatus. 5 gm of powdered leaves was taken in a cotton cloth and placed into flat-bottom beaker of the soxhlet containing 50 ml of 50% (v/v) ethanol and kept overnight. Soxhlated ethanolic extract so prepared was then transferred into Petri plates followed by their incubation at 37°C for 7-8 hours.

**Animal Groups**

Swiss mice (18-28 gms) of either sex were randomly divided into 5 groups of 6 mice each (Table 1). The mice were housed in a plastic cage at room temperature (25 ± 2°C) for the experiment.
### Table 1
Grouping of animal model.

| Group | Description                                      |
|-------|--------------------------------------------------|
| A     | Control                                          |
| B     | Smoke-induced                                    |
| C     | Dexamethasone-induced smoker                     |
| D     | Neem extract (100mg/kg BW) induced smoker        |
| E     | Neem extract (400mg/kg BW) induced smoker        |

#### Preparation of Drugs

The stock solution of dexamethasone was prepared by dissolving 1 mg of it in 1 ml of distilled water of which 250 µl was used as a working solution. The ethanolic neem leaf extract was used in two different concentrations which were 400 mg/kg body weight and 100 mg/kg BW in two mice groups respectively (smoker and non-smoker) shown in Table 1.

#### Smoke exposure

Mice were placed in a smoke chamber (a rectangular box) with a partition wall dividing it into two halves, containing 16 holes in it. A cigarette puff plugged into a motor was adjusted on either side of the box for its ignition whose smoke was allowed to pass through these small holes towards the other side of the wall where mice were placed. Cigarette Smoke exposure was continued for 5-7 minutes after the drug administration which was given 45 minutes before the CS exposure. This treatment was given thrice in a week with an interval of two days and continued for 7 weeks.

#### Administration of drug into the animal model

Drugs from working solution were administered into mice intraperitoneally 45 minutes before cigarette smoke exposure. This was repeated thrice a week for 7 weeks with an interval of two days.

#### Phytochemical Screening

**Test for tannins**

2 ml of neem leaf extract was taken to which 2 ml distilled water was added followed by introduction of few drops of Ferric chloride to confirm the presence of tannins. Green precipitates showed the presence of tannins (Sivakumar and Gajalakshmi 2013).

**Test for Saponins**

Distilled water and neem leaf extract were added into test tube in 1:1 ratio and shaken vigorously. The persistence of foam indicated presence of saponins (Ijoma et al. 2017).

**Test for sterols**
1 mg of crude neem leaf extract was dissolved into 10 ml of chloroform followed by the addition of an equal volume of conc. sulphuric acid through wall of test tube. The upper layer turned red while the lower layer appeared yellowish in colour with green fluorescence (Saklani et al. 2012).

Test for detection of protein

Concentrated Nitric acid (HNO₃) was added to neem leaf extract. The appearance of yellow colour confirmed the presence of proteins (Sati and Kumar 2015).

Test for phenols

Few drops of Ferric chloride (FeCl₃) were added to the extract. Formation of bluish-black precipitates showed the presence of phenols (Ijoma et al. 2017).

Test for alkaloids

The extract was dissolved into diluted HCl. Subsequently the mixture was filtered and later few drops of Wagner’s reagent were added. Reddish-brown precipitates at the bottom of test tube indicated the presence of alkaloids (De et al. 2010).

Test for flavonoids

A few drops of NaOH solution were added to few ml of neem leaf extract followed by the addition of diluted acid. Intense yellow colour was observed on the addition of NaOH which turned colourless on the addition of diluted acid (Mohammed 2019).

Test for carbohydrate

5 ml of distilled water was added to neem leaf extract and the mixture was then subjected to filtration through Whatman filter paper No. 1. Benedict’s solution was added to the filtrate so obtained. Orange-red precipitates gave positive results (Nadhiya et al. 2019).

Test for diterpenes

Neem leaf extract was added with water and few drops of freshly prepared copper acetate solution. Emerald green precipitates at the bottom of test tube confirmed the presence of diterpenes (Shahverdi et al. 2019).

Test for triterpenes

Chloroform was added to the neem leaf extract and mixture was then filtered. Conc. Sulphuric acid was added to the filtrate. Golden-yellow precipitates appeared in the test tube as per the method (Kumar et al. 2015).

Analysis of Bodyweight of mice
The body weight of mice was noted every week to observe gain/reduction due to the administration of dexamethasone, neem extract, and cigarette smoke. It was observed from the first to the last day of the experiment. The differences in body weights of mice were calculated before sacrificing them.

**Analysis of Mortality rate**

Mortality rate was observed during the whole experiment.

**Estimation of eosinophil peroxidase (EPO) in liver**

100 mg of mice tissue was homogenized (1:1) into 1.9 ml PBS followed by its centrifugation at 12000 X g for 10 minutes. Supernatant was taken for enzymatic assay and equal amount of substrate (0.075 mmol/L Tris- HCl pH 8, 1.5 mmol/L O- phenylenediamine, and 6.6 mmol/L H$_2$O$_2$ in) was added. Further reaction was stopped with the addition of 50 µL of 1mol/L H$_2$SO$_4$, and absorbance was recorded at 490 nm (Adamko et al. 2004).

**Histopathology of liver**

The histopathology of liver was performed as per the protocol (Ragavan et al. 2006). Tissue from liver was dissected and placed into 10% NBF (Neutral Buffered Formalin) followed by its dehydration using different grades of alcohol which was transferred to the cassette filled with filtered paraffin wax and left for whole day. After microtomy, tissue was fixed on slides with DPX (Distyrene, Plasticiser, and Xylene) to observe under microscope.

**Total Protein Content in liver tissue**

Total protein concentrations were measured as µg/ml in serum by Folin's assay (Lowry's method) as per the previously defined procedure (Waterborg et al. 1994). Reagents were added into samples and observed using UV-spectrophotometry.

**Liver function test by quantifying AST and ALT in serum**

5 µl serum was diluted upto 10 µl with distilled water. A reagent mixture was prepared from Autospan liquid gold standard kit (Arkray Healthcare Pvt. Ltd.) and ELISA was performed for the estimation of AST and ALT. 10 µl of serum as a sample was mixed in 100 µl reagent prepared according to assay kit [SGPT (DST) (IFCC Method) Rechon Diagnostics P. Ltd.] and ELISA was performed followed by its observation at 390 nm. The enzyme activity is expressed as International Units/Litre (IU/L).

**Estimation of Reactive Oxygen Species (ROS) in the liver**

Tissue homogenate (1:1) was prepared as per method (Shin et al. 2016) and was centrifuged followed by the addition of PBS and DCFDA to the pellet. Observations were taken at an absorbance of 485 nm and emission at 535 nm.

**Estimation of superoxide dismutase (SOD)**
Phosphate Buffer was added for the homogenization of tissue and other reagents were mixed including L-methionine, hydroxylamine hydrochloride, hydrogen peroxide, and EDTA followed by incubation for 10 mins under the white fluorescent light inside an aluminum foil-wrapped box. Freshly prepared Griess reagent was added and absorbance was measured at 543 nm (Das et al. 2006).

**Statistical analysis**

All the experiments were repeated twice to compare results. Experimental data were reported as mean ± SEM of n= 5. Multiple group comparisons were determined using Student’s t-test.

**Results**

**Phytochemical analysis of an ethanolic extract of** A. indica

Phytochemical investigation of *Azadirachta indica* ethanolic leaf extract revealed the presence of tannins, sterols, proteins, alkaloids, flavonoids and diterpenes while saponins, phenols, triterpenes and carbohydrates were found to be absent (Table 2). The presence of these biologically active compounds in leaf extract of *A. indica* probably holds the accountability for its anti-inflammatory property.

**Bodyweight analysis**

Bodyweight of mice was analyzed for 7 weeks after their treatment with dexamethasone and two different doses of *A. Indica*, viz 100 mg and 400 mg (Fig. 1). During first week, weight of mice remained constant in control, smoke-induced and in dexamethasone administered group as well. However, a subtle weight decline was observed during fourth week in CS-induced mice group while dexamethasone treated mice group resulted in a gradual weight reduction during second and fourth week. Additionally, body weight of mice was decreased during first week in both ethanolic neem extract induced groups, while further it remained constant throughout the experiment.

**Table 2.** Phytochemical analysis of ethanolic extract from the leaves of *Neem (Azadirachta indica)*
### Mortality Rate analysis

The mortality rate was observed weekly among all the mice groups during experiment (Fig. 2). Dexamethasone treated group exhibited 20% mortality rate in the fifth week of experiment while no mortality was observed in rest of the mice groups.

### Eosinophil peroxidase activity (EPO)

EPO activity was analysed to examine the recruitment and activation of eosinophil peroxidase. As illustrated in Fig. 3, the activity and level of eosinophil peroxidase elevated in the smoke-induced group of mice with 1.99 absorbance units when compared to control group. In parallel, dexamethasone administered group also showed rise in EPO activity at 1.97 absorbance units which was comparable to smoke-induced group while *A. indica* leaf extract treated group exhibited least EPO activity of 1.09 absorbance units.

### Histopathology of Liver

Liver tissues of mice from all the groups were sectioned appropriately and stained with Hematoxylin-Eosin staining. The histological images presented pathological variations in CS-induced and dexamethasone treated mice tissues (Fig. 4B and 4C respectively) when compared to normal tissue from control mice group (Fig. 4A). Cellular inflammation and disrupted architecture were seen in altered liver tissues of mice. On contrary, liver tissues from the mice group treated with 100 mg dose of ethanolic neem leaf extract showed reduced inflammatory cells (Fig. 4D) and furthermore, tissues from the mice group treated with 400 mg herbal dose resulted in least cellular inflammation and disruption, thereby tissue recovery (Fig. 4E).

### Estimation of total protein content in liver

| Bioactive Compounds | Present (+)/ Absent (-) |
|---------------------|-------------------------|
| Tannins             | +                       |
| Saponins            | -                       |
| Sterols             | +                       |
| Proteins            | +                       |
| Phenols             | -                       |
| Alkaloids           | +                       |
| Flavonoids          | +                       |
| Diterpenes          | +                       |
| Triterpenes         | -                       |
| Carbohydrates       | -                       |
Protein estimation in the blood serum was carried out in consequence of the proteinaceous inflammatory markers. As shown in Fig. 5, the protein content in the smoke-induced group of mice was higher being 15.82μg/mg compared to the control group, while it was reduced in the group administered with dexamethasone with the value of 11.99μg/mg yet it was elevated to 14.11μg/mg in 400 mg neem extract treated group. Probably, there was formation of protective proteins in mice administered with a high and low dose of neem extract.

**Liver Function Markers**

Aspartate aminotransferase (AST) activity

Effects of tobacco smoke, dexamethasone and leaf extract of *A. Indica* on the activity of aspartate aminotransferase (AST) was analysed. The examination revealed a higher serum AST activity in the smoke-induced group at 68.00 IU/L in comparison to the control group of mice. AST level in dexamethasone treated mice group, 56.00 IU/L, was comparable to the mice group treated with cigarette smoke while the activity of AST was minimum in 400 mg herbal extract treated group at 10.2 IU/L (Fig. 6).

Alanine aminotransferase (ALT) activity

Alanine aminotransferase (ALT) activity was investigated to check the impacts of neem leaf extract. The results demonstrated higher activity of ALT in the smoke-induced and dexamethasone treated group at 72.50 IU/L and 64.90 IU/L respectively as compared to the normal group, while serum ALT was highly reduced in 400 mg dose of neem leaf extract with 15.90 IU/L level, as shown in Fig. 7.

**Estimation of Reactive Oxygen Species (ROS) in mice liver**

Reactive Oxygen Species (ROS) were analysed to examine the free radical generation during smoke-induced inflammation and the effect of neem extract on it. ROS were found to be the maximum in the smoke-induced group of mice at 34600 RFU (relative fluorescence unit) when compared with control group. Dexamethasone induced group showed a prevalence in the number of ROS at 32500 RFU whereas the group treated with 100 mg dose of leaf extract resulted in reduced ROS levels at 22400 RFU. Furthermore, the group treated with 400 mg dose showed the least amount of ROS at 21900 RFU (Fig. 8).

**Estimation of Superoxide Dismutase (SOD)**

Superoxides are produced as by-product of oxygen metabolism and, if not regulated, can cause cell damage. Superoxide dismutase (SOD) constitutes a vital antioxidant defense against oxidative stress in the body. As illustrated in Fig. 9, the superoxide dismutase activity in this study was maximum in control mice as 7.50 n kat/mg. The SOD activity declined in smoke-induced mice which was recovered on treatment with 400 mg dose of neem leaf extract upto the level of 7.17 n kat/mg. Dexamethasone treated group also resulted elevation in SOD activity to a level of 4.30 n kat/mg, yet it was not comparable to herbal extract.
Discussion

Smokers are at considerable risk of cardiovascular diseases (ischaemic heart disease, hypertension), respiratory disorders (bronchitis, emphysema, chronic obstructive lung disease, asthma), cancer (lung, pancreatic, breast, liver, bladder, oral, larynx, oesophagus, stomach, and kidney), peptic ulcers and gastroesophageal reflux disease (GERD), male impotence and infertility, blindness, hearing loss, bone matrix loss, and hepatotoxicity. Cigarette smoke is responsible for a variety of adverse and hazardous effects on organs which do not have direct exposure to it such as liver and that’s way it is prone to toxic immunological and oncogenic effects (El-Zayadi 2006). It yields numerous chemicals that are structurally distinct involving free radicals having cytotoxic potential and are responsible for increase in necro-inflammation and fibrosis. Moreover, tobacco smoke contributes in the development of secondary polycythemia which might be a contributing factor to secondary iron overload disease-promoting oxidative stress of hepatocytes (Gutteridge et al. 1989).

In the present study, effects of neem extract and dexamethasone on cigarette smoke induced hepato-injury were analysed. Leaf extract of A. indica (100 mg/kg and 400 mg/kg) was administered into mice which had anti-inflammatory and antioxidative role in treating liver-injury (Biswa et al. 2002). The phytochemical screening of leaf extract of A. indica revealed the presence of several bioactive compounds involving tannin, saponin, sterols, protein, phenols, alkaloids, flavonoids, diterpenes, triterpenes, and carbohydrates (Vinoth et al. 2012). Although, flavonoid is a large group of naturally occurring compounds that exhibits anti-inflammatory and antioxidant activity, so far terpenes and saponins also get hold of some anti-inflammatory activity (Sultana et al. 2017). Within realm of possibility, the phytochemicals present in A.indica were accountable for manifesting anti-inflammatory as well as antioxidant property in cigarette smoke-induced liver injury as demonstrated in the animal model. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were found to be commonly used biomarkers for liver damage (Alsalhen et al. 2014). However, presence of AST also in cardiac muscle, skeletal muscle and erythrocytes obliged ALT to be the most specific marker for liver damage (Goorden et al. 2013). In this study, the levels of AST and ALT were examined for the functioning of the liver where cigarette smoke caused liver injury was indicated by the elevated levels of AST and ALT. The mice group treated with dexamethasone resulted in lower levels of AST and ALT yet it was not comparable to the A. Indica extract treatment which showed a highly diminished levels of AST and ALT suggesting that the liver damage was controlled in more proportion on administration of A. indica. The inflammation was confirmed by observing the enhanced activity of EPO, a marker of eosinophilic inflammation (Saidani et al. 2019). In this study, the activity of EPO enhanced in smoke-induced mice was suppressed on administration of A. indica extract at both doses and perhaps the bioactive compounds of A. indica were responsible for the anti-inflammatory effect. Besides, another inflammatory marker was protein concentration which shared similar results as that of EPO. An advanced antioxidant system has been developed to relieve oxygen stress, however an excessive amount of reactive oxygen species (ROS) disrupts homeostasis which develops oxidative stress that ultimately leads to hepatic disorders by bringing irremediable changes in lipids, proteins and DNA content (Li et al. 2015). Redox state constitutes an essential background for numerous liver disorders owing the fact that it is associated with the course
of inflammatory, metabolic, and proliferative liver diseases (Cichoz et al. 2014). In our investigation, generation of ROS was found to be elevated in the smoke-induced group providing the basis for inflammation in liver tissues. Furthermore, ROS production in this study was enhanced by the suppressed activity of superoxide dismutase (SOD) as observed in liver tissues of *Labeo rohita* during toxicity test (Bojan et al. 2017). The enzyme served as an anti-inflammatory agent and also prevented precancerous cell changes (Karimi et al. 2017). The analysis of SOD activity resulted in the inhibition of ROS generation on treatment with *A. indica* extract demonstrating the anti-inflammatory and anti-oxidative function of herbal dose.

**Conclusions**

Corticosteroids are anti-inflammatory drugs used to treat numerous conditions. Dexamethasone is one of them that have side effects on body organs. Herbal approach could be its best alternative being a source of medicines from the ancient times. The current investigation demonstrates that the doses (100mg and 400mg) of ethanolic leaf extract of *Azadirachta indica* are relatively protective than dexamethasone in order to cure hepato-injury caused by cigarette smoke on account of no mortality and no severe toxic effects appeared in model organism on its administration based on the biochemical parameters and histopathology. Elicited from this research it can be concluded that *A. indica* have high margin of safety than dexamethasone in treating cigarette smoke induced liver damage yet further studies with different doses of extract are needed.

**Declarations**

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**Competing interest** The authors declare no competing interest.

**Author contribution** Subhashini suggested and designed the study. Anushree Verma performed the research experiments and analysis including extract preparation, phytochemical screening, biochemical and histopathology evaluation. Yashasvi Khajuria wrote the original manuscript and Vishal Srivashtav edited the final manuscript.

**Ethical approval** All procedures performed in studies involving animals were in accordance with the ethical standards of Central Animal Ethical Committee, Banaras Hindu University, Varanasi, India.

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Figures

Figure 1

Graphical representation of the effect of the synthetic drug and herbal extract on the bodyweight of the model organism.
Figure 2

Effect of a synthetic and herbal drug on the life-span of mice.

Figure 3

Effect of Dexamethasone and *A. indica* leaf extract on Eosinophil peroxidase activity in smoke-induced liver injury.
Figure 4

Morphology of liver tissue by Haematoxylin & Eosin staining; A. Control mice liver; B. Highly disruption observed in smoke-induced mice liver; C. Cells disruption and the presence of inflammatory cells were found in Dexamethasone-induced mice liver; D. Reduction in inflammatory cells was found in low dose neem treated mice liver; E. Damage recovery in high dose neem leaf extract induced liver.

Figure 5
Effect of Dexamethasone and *A. indica* on the protein content in the serum of a smoke-induced group of mice.

**Figure 6**

Effect of Dexamethasone and *A. indica* on aspartate aminotransferase (AST) activity in serum of smoke-induced mice.
Figure 7

Effect of Dexamethasone and *A. indica* on alanine aminotransferase (ALT) activity in serum of smoke-induced liver injury.

Figure 8
Effect of Dexamethasone and *A. indica* on ROS production in serum of smoke-induced liver injury.

![Graphical Abstract](Image)

**Figure 9**

Effect of Dexamethasone and *A. indica* on SOD activity in smoke-induced liver injury.

**Supplementary Files**

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- [GraphicalAbstract.tif](Image)