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Research Article

Anti-inflammatory and cytotoxicity assay of Cardamom-based nutraceuticals in comparison with 1,8 -Cineol

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Abstract

Aim: To investigate anti-inflammatory, cytotoxic effects and viability of Recovereez- 51% 1,8-Cineol and 1,8-Cineol (natural), and to determine safety for both of the compounds and its use in new product range of nutraceuticals.

Methodology: Cell line was divided into two groups; one was treated with Recovereez(R) and another one with 1,8-Cineol(C). The ELISA assay method was used to measure the COX, LOX, MPO, NO, and nitrate levels. Cellular viability was performed by observing cells under the inverted phase contrast microscope, followed by MTT assay.

Results: R&C showed a reduction in levels of inflammatory markers such as IL-6, IL-1β, TNF-α, LOX, MPO, COX, iNOS, and nitrate levels. The inhibition as observed in the levels of COX and LOX enzymes were 65%±0.1 and 59%±0.3µg/ml, and 72%±0.3 and 67%±0.4µg/ml for R&C, respectively. iNOS production showed inhibition by 70%±0.3 and 65%±0.8µg/ml and NO production were reduced by 193 and 209.5µg/ml by R&C, respectively. Again, there was a decrease in the activity of MPO by 0.35 and 0.39U/ml for R&C, respectively. The results were significant with $p<0.001$ compared to the Diclofenac sodium. Cell viability of 60% was observed upon treating L929 cells with R&C.

Conclusion: R&C exerted an anti-inflammatory effect on RAW 264.7 cells without exerting cytotoxicity in a dose-dependent manner. Therefore, Recovereez could be used as an alternative treatment or to prevent inflammatory diseases. Moreover, R&C are expected to treat different types of cancer. The findings could potentially lead to the discovery of safe and effective bioactive compounds that can prevent or cure the occurrence of cancerous cells.

Keywords: dietary supplements, nutraceutical, COX, anti-inflammation, cancer

INTRODUCTION:

Elettaria cardamomum or green cardamom is a unique aromatic spice, popular for its common use in Indian households as spice [1]. A lot of nutraceutical-related work has been performed on curcumin from turmeric and has received substantial attention due to its nutritional values and therapeutic effects, with fewer side effects. Ayurvedic literature studies suggest the use of Cardamom and its natural extracts in disease management thus leading to the development of modern therapeutics [2]. The World Health Organization (WHO) promotes the use of plants with scientific research for the therapy of systemic diseases [3]. Cardamom extracts are acknowledged for their strong antioxidant properties and as targets for important molecular mechanisms in natural and synthetic medicines [4]. One such therapeutically important phyto-compound is 1,8-Cineol and cardamom acts as a good source of 1,8-Cineol. Presently, this clinically significant compound is less studied and has fewer therapeutic applications. Periodontitis and dental caries of the oral cavity need special attention attributing to their epidemic situation [5]. The current study on the anti-bacterial activity of green cardamom extracts has focused on its therapeutic use against periodontal infections and the protection of the oral cavity [6]. 1,8-Cineol regulates the inflammatory reaction cascade of NF-κB by involving a reduction in levels of reactive oxygen species (ROS) linked- oxidative stress and by decreasing the enhanced levels of IL-1β and TNF-α [7]. Many pre-clinical studies have demonstrated the anti-cancerous effects of Cardamom essential oil through its cytotoxic, antioxidant, and anti-inflammatory properties. Moreover, several other studies also show that other phytochemicals present in the cardamom plant are also known to impart anti-oxidative, anti-microbial, and anti-hypertensive properties [8, 9].

Recovereez is a proprietary formulation available as a capsule constituent of cardamom extract at 200mg. rosemary extract at 200mg; and pepper extract at 10mg. It is primarily the active bioingredient 1,8- Cineole, constituting 97 mg per capsule. Clinical studies have demonstrated considerable anti-inflammatory action is proven by the comparative analysis of the gene expression profile of inflammatory markers with regularly tested inflammatory parameters such as serum IL-6, TNF- α, etc. [10]. The persistent anti-inflammatory action of Recovereez is assessed as equivalent to steroids as it calms the rise in inflammation and ameliorates the associated symptoms of the body.

Distributionally, much of the world's Cardamom is produced in Guatemala in South America, the second largest producer being India [1]. In India, Kerala is the largest cardamom producer accounting for 70% of its total production alone,

which is followed by states like Karnataka (20%) and Tamil Nadu (10%) [11].

This naturally-derived monoterpene compound, 1,8-Cineole is chemically a terpenoid oxide, the major constituent (nearly 70%) of essential oils procured from various eucalyptus species [12]. Several studies have shown that 1,8- Cineol, acts as an anti-inflammatory mediator, and mucociliary inducer that significantly helps in alleviating respiratory disorders like asthma and COPD [13]. 1,8-Cineole acts as a modulator of the physiological pathways of COPD and thus, bring relief to its associated pathology and symptoms [14]. 1,8-Cineole exerts a similar inhibitory effect on inflammation resulting from cyclooxygenase (COX-1, COX-2) activity which is compared with celecoxib, a COX-2 inhibitory drug, and indomethacin, a nonsteroidal anti-inflammatory drug (NSAIDs) [15]. Another study that involves the use of Eucalyptus oil, majorly composed of 1,8-Cineole has revealed lipopolysaccharide (LPS)-induced inflammatory signaling pathways altering the enhanced levels of TNF-α, IL-1 (α and β), and NO in the lung by alveolar macrophages [16]. We aim to establish the efficacy and safety of the formulation, Recovereez which contains 51% 1, 8- Cineol which is comparatively more efficient than the pure compound alone owing to its anti-inflammatory role and cytotoxicity. In addition, as our formulation is derived from standardized natural extracts, the incidence of other ingredients that influences the claims of anti-inflammatory action does exist. Nevertheless, this possibility can be well distinguished against the active ingredient 1,8-Cineole, when used alone. A comparative cytotoxicity assay will help to establish the safety of the use of Recovereez as a nutraceutical with natural anti-inflammatory agents.

METHODS:

RAW 264.7 cells were initially procured from National Centre for Cell Sciences (NCCS), Pune, India, and maintained in Dulbecco's Modified Eagle's Medium, DMEM (Sigma Aldrich, USA). The cell line was cultured using a 25 cm^2 tissue culture flask containing DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany), and antibiotic solution of Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5 µg/ml). The cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). The cells were grown to 60% confluency followed by their activation with 1 µL lipopolysaccharide (LPS: 1 µg/mL). LPS-stimulated RAW 264.7 cells were exposed to different concentrations (25, 50, 100 µg/mL) of sample solution and Diclofenac sodium, a standard anti-inflammatory drug, in varying concentrations corresponding to the sample, and incubated for 24 hours. Following the incubation period, the anti-inflammatory assays were performed using the cell lysate.

Cyclooxygenase (COX) activity

The COX activity was assayed by utilizing the method of Walker and Gierse [17]. 100µl cell lysate was incubated with Tris-HCl buffer (pH 8), glutathione 5 mM/L, and hemoglobin 5 mM/L for 1 minute at 25°C. The reaction was initiated by the addition of arachidonic acid 200 mM/L and terminated after 20 minutes of incubation at 37°C, by the addition of 200 µL of 10% trichloroacetic acid in 1 N hydrochloric acid. After the centrifugal separation and the addition of 200 µL of 1% thiobarbiturate, the tubes were boiled for 20 minutes. After cooling, the tubes were centrifuged for three minutes. COX activity was determined by reading the absorbance at 632 nm.

Lipoxygenase (LOX) activity

The determination of LOX activity was done as per Axelrod *et al.* [18]. Briefly, the reaction mixture (2 mL final volume) contained Tris-HCl buffer (pH 7.4), 50 µL of cell lysate, and sodium linoleate (200 µL). The LOX activity was monitored as an increase in absorbance at 234 nm (Shimadzu), which reflects the formation of 5-hydroxyeicosatetraenoic acid.

Myeloperoxidase (MPO) activity

The cell lysate was homogenized in a solution containing 50 mM potassium phosphate buffer and 0.57% hexadecyltrimethylammonium bromide (HTAB) the samples were centrifuged at 2000 g for 30 minutes at 4°C, and the supernatant was assayed for MPO activity. MPO in the sample was activated by the addition of 50 mM phosphate buffer (pH 6) containing 1.67 mg/mL guaiacol and 0.0005% H_2O. The change in absorbance at 460 nm was measured. MPO activity was presented as units per mL of cell lysate. One unit of MPO activity was defined as the degradation of 1 µM of peroxide per minute at 25°C.

Inducible Nitric Oxide Synthase

Nitric oxide synthase was determined by the method described by Salter *et.al* 1996 [19]. The cell lysate was homogenized in 2 ml of HEPES buffer. The assay system contained substrate 0.1ml -2 µmol/L L-Arginine, 0.1ml- 4 µmol/L manganese chloride, 0.1ml-10 mmol/L 30 µg dithiothreitol (DTT), 0.1 ml- 1 mmol/L NADPH, 0.1 ml- 4 µmol/L tetrahydropterin, 0.1 ml 10 µmol/L oxygenated hemoglobin and 0.1 ml enzyme (sample). An increase in absorbance was recorded at 401 nm.

Estimation of Cellular Nitrite Levels

The level of nitrite level was estimated by the method of Lepoivre *et al*. [20]. To 0.5 mL of cell lysate, 0.1 mL of 3% sulphosalicylic acid was added and vortexed well for 30 minutes. The samples were then centrifuged at 5,000 rpm for 15 minutes. The protein-free supernatant was used for the estimation of nitrite levels. To 200 µL of the supernatant, 30 µL of 10% NaOH was added, followed by 300 µL of Tris-HCl buffer, and mixed well. To this, 530 µL of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% N-1-naphthyl ethylene diamine dihydrochloride) was added and incubated in the dark for 10–15 minutes, and the absorbance was read at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained.

In Vitro cytotoxic effect determination by MTT assay

L929 (Fibroblast) cells were initially procured from National Centre for Cell Sciences (NCCS), Pune, India, and maintained in Dulbecco's Modified Eagle's Medium, DMEM (Sigma Aldrich, USA). The cell line was cultured in a 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-Glutamine, sodium bicarbonate (Merck, Germany), and antibiotic solution containing: Penicillin (100 U/ml), Streptomycin (100 µg/ml), and Amphotericin B (2.5 µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). The viability of cells was evaluated by direct observation of cells by an inverted phase contrast microscope and followed by the MTT assay method.

Cell seeding in 96-well plate:

Two-day old confluent monolayer of cells was trypsinized and the cells were suspended in 10% growth medium, 100 µl cell suspension (5x103 cells/well) was seeded in 96-well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Preparation of compound stock:

1 mg of the sample was weighed and dissolved in 1 mL DMEM using a cyclomixer. The sample solution was filtered through a 0.22 µm Millipore syringe filter to ensure sterility.

Cytotoxicity evaluation:

After 24 hours the growth medium was removed and the freshly prepared each compound in DMEM was five times serially diluted by two-fold dilution (100 µg, 50 µg, 25 µg, 12.5 µg, 6.25 µg in 500 µl of DMEM), and each concentration of 100 µl were added in triplicates to the respective wells and incubated at 7ºC in a humidified 5% CO_2 incubator. Non-treated control cells were also maintained.

Cytotoxicity Assay by Direct Microscopic Observation:

The entire plate was observed after 24 hours of treatment in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observations were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation, and vacuolization in the cytoplasm of the cells were considered indicators of cytotoxicity.

Cytotoxicity Assay by MTT Method:

15 mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells was removed and 30 µl of reconstituted MTT solution was added to all test and cell control wells. The plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (Dimethyl sulphoxide: DMSO, Sigma Aldrich, USA) was added and the wells were mixed gently by pipetting up and down to solubilize the formazan crystals. The absorbance values were measured by using a microplate reader at a wavelength of 540 nm (Laura B. Talarico *et al.,* 2004) [21].

Statistical Analysis:

Data are expressed as the mean ± Standard deviation (SD) from three independent experiments. Differences between the sample and LPS groups were determined by One way Analysis of Variance (ANOVA), followed by Dunnett's Multiple comparison test. Differences were considered significant at p<0.005. The statistics program was calculated using ED50 PLUS V1.0 Software.

RESULTS:

The results of COX inhibitory activity are presented in Fig 1. Results indicate that for both the natural and synthetic drugs, the highest inhibitory activity observed is comparable to the standard Diclofenac sodium, a standard anti-inflammatory drug.

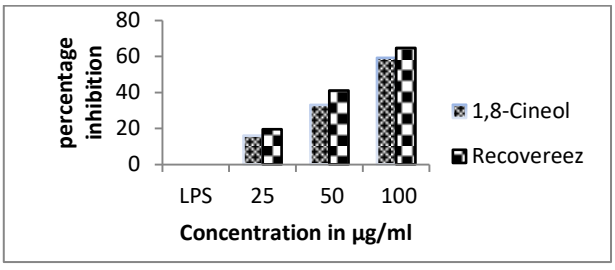

Figure 1: Effect of 1, 8- Cineol and Recovereez on COX expression in LPS-stimulated RAW 264.7 cells. The data are presented as mean ± standard deviation (SD) of at least three independent experiments. p< 0.001 indicated significance compared to the LPS-treated group

Recovereez exhibits COX inhibitory activity of 65% ± 0.1 µg/ml at a concentration of 100 µg/ml, followed by 1, 8- Cineol which shows 59% ± 0.3 µg/ml cell inhibition at a concentration of 100 µg/ml. As observed from the above experiment, the cell activity is inhibited in a dose-dependent manner. The results are significant at p<0.001 when compared to the Diclofenac sodium drug. The LOX inhibitory activity is 72% ± 0.3 µg/ml and 67% ± 0.4 µg/ml by Recovereez and 1,8 -Cineol, respectively (Fig 2).

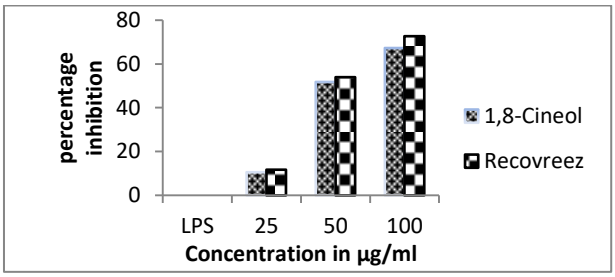

Figure 2: Effect of 1,8 -Cineol and Recovereez on LOX expression in LPS-stimulated RAW 264.7 cells. The data are presented as mean ± standard deviation (SD) of at least three independent experiments. p< 0.001 indicated significance compared to the LPS-treated group

iNOS is known as a key player in the synthesis of NO production during inflammation. The results of our study showed the highest range of inhibition by Recovereez 70% ± 0.3 µg/ml while compared to 1, 8- Cineol 65% ± 0.8 µg/ml. The LPS-induced cells reveal zero percentage inhibition. The results are significant with p-value (p<0.001). (Fig3)

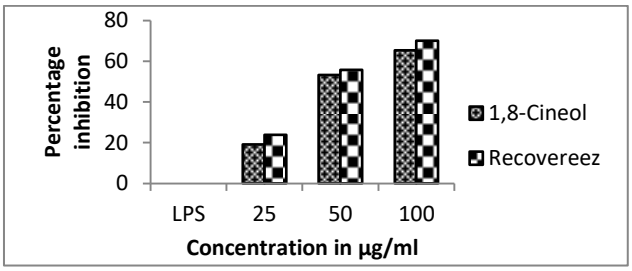

Figure 3: Effect of 1,8-Cineol and Recovereez on iNOS expression in LPS-stimulated RAW 264.7 cells. The data are presented as mean ± standard deviation (SD) of at least three independent experiments. p< 0.001 indicated significance compared to the LPS-treated group

The effect of 1,8 -Cineol and Recovereez on the generation of NO, a critical mediator of the inflammatory response is examined. RAW 264.7 cells are treated with LPS, 1, 8- Cineol, and Recovereez as described above and the generated NO is quantified as nitrite using Griess assay. NO production is decreased at a concentration of 100 µg/ml by 193 µg/ml and 209.55 µg/ml by Recovereez and 1,8 -Cineol, respectively (Fig 4). There is a decrease in the production of NO as the concentration of Recovereez and 1, 8- Cineol are increased. This confirmed the potent inhibitory activities of 1, 8- Cineol, and Recovereez on NO production and its potential to inhibit iNOS expression.

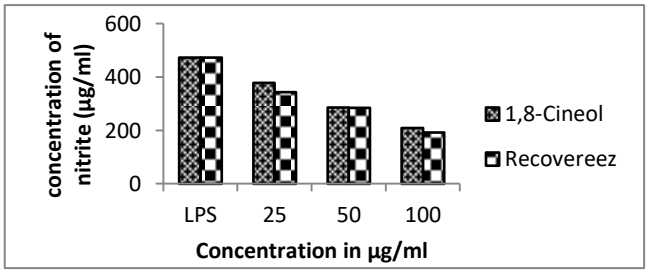

Figure 4: Effect of 1, 8- Cineol and Recovereez on NO production in LPS-stimulated RAW 264.7 cells. The data are presented as mean ± standard deviation (SD) of at least three independent experiments. p< 0.001 indicated significance compared to the LPS-treated group

MPO represents the most abundant proinflammatory enzyme whose release may be associated with the pathogenesis of several diseases. In our study there is a decrease in the MPO activity of 0.35 U/ml by Recovereez and 1,8 -Cineol showed 0.39 U/ml of MPO activity, whereas the LPS-induced sample showed 0.53 U/ml of activity (Fig 5).

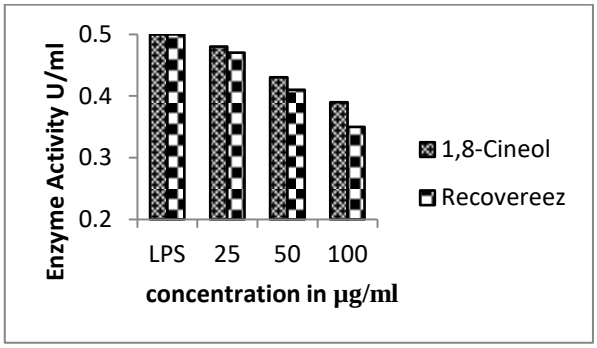

Figure 5: Effect of 1,8 -Cineol and Recovereez on MPO activity in LPS-stimulated RAW 264.7 cells. The data are presented as mean ± standard deviation (SD) of at least three independent experiments. P< 0.001 indicated significance compared to the LPS-treated group

The cytotoxicity effects of 1, 8- Cineol and Recovereez are depicted in Fig 6a, 6b. In our study, a series of different concentrations of 1,8 -Cineol and Recovereez are designed to observe a possible dose-dependent relationship. According to the result, as the dilutions increased, the amount of cell viability also increased, thereby showing that the diluted extract concentration proved to be less toxic. Recovereez and 1,8 -Cineol almost showed the same viability at 98% (Fig 6).

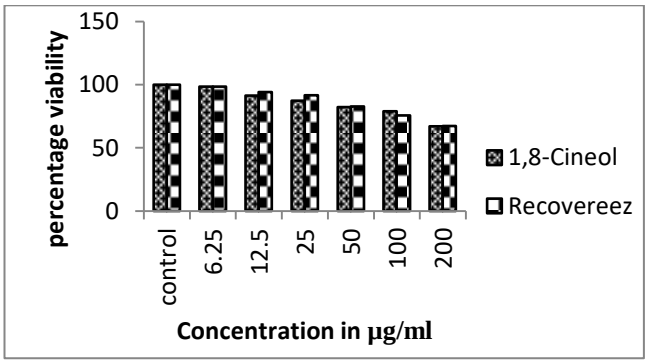

Figure 6: Effect of 1,8-Cineol and Recovereez on cell viability of RAW 264.7 cells. The data are presented as mean ± standard deviation (SD) of at least three independent experiments. P< 0.001 indicated significance compared to the control group

DISCUSSION:

The natural antioxidant defense system is a highly complex biochemical organization that consists of numerous enzymes and a large number of scavenger molecules [22]. The increase in the prevalence of multiple drug resistance has resulted in the search for new anti-inflammatory cures from alternative sources [23]. Currently, there is a significant surge in the search for new drug derivatives from natural extracts possessing anti-inflammatory properties that are already in trend for the treatment of various chronic and infectious diseases. The development of inflammation is the genesis of many disease states, including headache, atopy, metabolic syndrome, multiple sclerosis, Alzheimer's disease, and Parkinson's disease [24-26].

LPS acts as a major constituent of the outer wall of the cells of gram-negative bacteria. It activates macrophages to induce the release of high concentrations of pro-inflammatory cytokines, and inflammatory mediators including TNF-α [27]. The cell-killing activity of macrophages is majorly due to the incitation of TNF pathways [28]. Here, the cell viability studies performed denote the enhanced cytotoxicity associated with an increase in the concentration of Recovereez and 1, 8- Cineol.

Previous papers suggested that phytochemicals possess anti-inflammatory activity by virtue of their highly correlated contents of phenolic acids, flavonoids, anthocyanidins, and anthocyanins [29- 31]. The anti-inflammatory effects are attributed to the extent of inhibition of the inflammatory reaction at different stages involving COX and LOX activity [32]. Arachidonic acid is the key metabolite in COX and LOX mediated- pro-inflammation cascades [33]. Therefore, both of these pathways can be anti-inflammatory targets of our formulation Recovereez. Cyclooxygenase (COX), present as two isoforms, COX-1 and COX-2, is one of the most significant enzymes in contribution to human pathophysiology. COX-2 is an inducible enzyme that catalyzes the biosynthesis of PGE2, which contributes to the pathogenesis of various inflammatory diseases. COX inhibition is the mechanism of action for most non-steroidal anti-inflammatory drugs [32]. Lipooxygenases (LOX) are involved in rise of leukotriene-mediated allergic inflammation which can prevent the root cause of many diseases including cardiovascular disorders and atherosclerosis [33]. In our study, Recovereez exhibited COX inhibitory activity of 65% ± 0.1 µg/ml followed by that of 1,8-Cineol at 59% ± 0.3 µg/ml at the concentration of 100 µg/ml. The LOX inhibitory activity was 72% ± 0.3 µg/ml and 67% ± 0.4 µg/ml by Recovereez and 1, 8- Cineol respectively, which indicates that Recovereez and 1, 8- Cineol impart a good anti-inflammatory activity to cells that inhibit the COX and LOX enzymes.

Inducible nitric oxide synthase (iNOS) mediates inflammation and is found to be induced by LPS which enzymatically generates the pro-inflammatory mediator, NO. Besides LPS, other cascades that intervene in NO production are IFN-γ and TNF- α [34]. An indigenously known, less explored plant *Borassus flabellifer* L. whose haustorium and kernel are known for their anti-inflammatory and cell-protective qualities, are shown to alleviate pro-inflammatory cytokines in LPS-induced RAW 264.7 cells [35]. Another pre-clinical model involving the lipophilic grape seed proanthocyanidin (LGSP) indicates significant depletion of immunogenic markers like NO, TNF- α, IL- 6, and IL- 1β. [36] The drug formulation from *Epigyneum auritum* extract reveals a significant drop in levels of inflammation mediators NO, ROS, TNF-α, and IL-6 in *in vitro* LPS-induced RAW264.7 macrophages. Besides it also diminished phosphorylated- levels of induced ERK1/2, JNK, and p38 MAPK, and decrease in nuclear translocation of NF-κB. Similar results are also observed in *in vivo* studies [37]. Tian Y *et al.,* studied that amber (a fossil resin) extract suppresses the increased TNF-α, IL-6, COX-2, and iNOS expression levels, thus indicative of inhibition of the NF-κB transcription pathway and also manages oxidative stress levels by reducing ROS and NO levels, in an LPS-induced experiment [10]. A study by Baek SH *et al.* showed that Sinapaldehyde exerts its anti-inflammatory effects by inhibiting iNOS and COX-2 protein expression, which was in accordance with our study [38]. Similarly, in a study by Eun-Jin Yang *et al*., NO production was decreased by 64% by using 200 µg/mL of *Sonchus oleraceus* extract (J6). J6 extract had a suppressive effect on the LPS-induced expression of iNOS in a concentration-dependent manner [39]. Therefore, these inflammatory pathways can be important targets of modulators of inflammation for Recovereez. Our study showed the highest range of iNOS inhibition by Recovereez at 70% ± 0.3 µg/ml when compared to the activity of 1, 8- Cineol at 65% ± 0.8 µg/ml. NO production was significantly reduced at a concentration of 100 µg/ml by 193 µg/ml and 209.55 µg/ml by Recovereez and 1, 8- Cineol respectively.

Immuno-pathogenic reactions generate oxidative stress levels that cause tissue injury at several intensities. Myeloperoxidase (MPO) represents an abundant pro-inflammatory enzyme whose release may be associated with the pathogenesis of several diseases [40- 42]. It has a vital role in atherosclerosis and plague deformation [43]. In our study there was a decrease in the MPO activity of 0.35 U/ml by Recovereez and 1,8 -Cineol showed 0.39 U/ml of MPO activity, significantly compared to the LPS-stimulated sample shows 0.53 U/ml of activity.

CONCLUSION:

This study convinced that Recovereez has novel anti-inflammatory activities in the LPS-induced RAW 264.7 cell model. 1,8-Cineol is the most abundant constituent of essential oils and has demonstrated good anti-inflammatory activity. Therefore, it is evident that Recovereez with a 51% composition of 1,8-Cineol carries greater potential as an anti-inflammatory drug than 1,8-Cineol used alone. The inhibitory activity of COX, LOX, and iNOS upon treatment with Recovereez is 65% ± 0.1 µg/ml, 72% ± 0.3 µg/ml, and 65% ± 0.8 µg/ml, respectively. Further, the NO production is decreased by 193 µg/ml. These results are comparable to figures of 1,8-Cineol that show the inhibitory activity of COX, LOX, and iNOS as 59% ± 0.3 µg/ml, 67% ± 0.4 µg/ml and 65% ± 0.8 µg/ml, respectively. NO production is decreased by 209.55µg/ml. Furthermore, 1,8 cineol and Recovereez are found to significantly attenuate the expression of the pro-inflammatory cytokines IL-6, IL1- β, TNF- α, and TGF- β and also effectively inhibit the expression of pro-inflammatory mediators COX, LOX, and iNOS, which in turn decreased the NO production. Recovereez statistically showed a synergistic activity that stands more effective than 1,8 -Cineol. The above results indicate that Recovereez can be used as an alternative treatment or in the prevention therapy of inflammatory diseases. Recovereez is expected to cure inflammation and different types of cancer. The novelty in Recovereez could potentially lead to the discovery of a safe and effective new bioactive drug that can prevent or cure cancer formation.

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Conflict of Interest:

The authors Of This Manuscript certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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